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A HEPATOGENOUS PHOTOSENSITIVITY DISEASE OF CATTLE: LESIONS, FUNCTIONAL DISTURBANCES AND BIOCHEMICAL ALTERATIONS

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CHAPTER I

INTRODUCTION

Photosensitivity in Domestic Animals

Diseases of domestic animals in which photosensitivity is one of the principle manifestations have a rather widespread distribution and occur in most of the major livestock producing areas throughout the world. These diseases have a tendency to be sporadic and usually have a transient course. Consequently, they are often relegated to a position of minor importance and receive only passing attention. However, they represent one of the major categories of disease affecting domestic animals in some areas and, at times, may be of greater economic significance than diseases of an infectious nature. This is particularly true in areas of New Zealand, South Africa and, to a lesser extent, in the southern and southwestern regions of the United States.

Photosensitivity diseases of domestic animals may be either hereditary or acquired. Those of hereditary origin are relatively uncommon. The hereditary photosensitivity diseases which have been recognized bear considerable similarity to certain of the porphyrias in humans and, therefore, have attracted greater attention than is probably warranted by their frequency of occurrence and economic importance to the

livestock industry. However, the need for continued investigation of the porphyrias in animals should not be minimized. Such cases have obvious value for comparative studies and as experimental models in further elucidation of mechanisms involved in disturbances in prophyrin metabolism.

The majority of photosensitivity disease syndrome in animals are acquired. They are caused either directly by photodynamic substances which enter the body in the absence of pre-existing lesions and functional disturbances, or indirectly, as a result of functional disturbances caused by toxic agents. There are indications that an increased incidence of photosensitization by the indirect mechanism is favored by the systems of husbandry and agricultural practices being adopted for more concentrated livestock production. This is particularly true in the southern and southeastern United States. Such changes in agricultural practices may lend increasing importance in the future to photosensitivity diseases which have received only limited attention in this country in the past.

Many of the reports concerning photosensitivity diseases consist of accounts only of the more obvious external manifestations. There is a discouraging paucity of critical clinical observations and supporting laboratory data. Assumptions and conclusions regarding the cause and genesis of the disease process which are based on such inadequate data are of limited value in comparative evaluation and appropriate classification of the particular photosensitivity disease.

Scope and Objectives

The present study is a continuation of earlier investigations of a hepatogenous photosensitivity disease of cattle. The preliminary investigations, initiated in 1958, were designed to establish the essential

nature of the disease with respect to the hepatic lesions and mechanism of development of photosensitization. The present investigation is designed to furnish additional experimental data for more complete characterization of the disease. Emphasis is placed on biochemical alterations in the blood serum and the relationship of these changes to the hepatic lesions and clinical signs.

History and Major Aspects of the Disease

During the fall and winter of 1957-58, an epizootic of photosensitization appeared in cattle in the eastern one-half of Oklahoma and in adjacent areas of the surrounding states. Prior to this time the occurrence of photosensitivity disease in this area was very low and sporadic. Following its first appearance in November, 1957, the rapidly increasing incidence of the disease throughout such a large area paralleled the pattern usually observed in explosive outbreaks of highly virulent infections. However, the clinical features of this photosensitivity disease did not suggest an infectious process.

The development of massive cutaneous lesions with typical propensity for the lesser pigmented areas of the body, together with obvious clinical signs of irritation in affected animals upon exposure to sunlight, readily established that the condition was a photosensitization. Lesions of the skin first developed and were more severe in the white or lightly-pigmented areas which were not protected by dense hair. Severely affected animals, however, did develop lesions in some of the more densely haired areas. Recognizable gross lesions were not observed in deeply pigmented skin. The constant occurrence of icterus in affected animals, together with the hepatic lesions observed at necropsy, suggested that the photo-

sensitization might be secondary to the hepatic lesions.

Observations made during the investigations of field outbreaks suggested that the disease was associated with the feeding of flood-damaged alfalfa hay. This association was not readily apparent in the first cases because alfalfa hay is such a common livestock feed in this area and there were no known reports incriminating alfalfa hay as a cause of photosensitization. Though the results of the early field investigations have been reported (Glenn, 1961; Monlux et al., 1963), a resume of the findings seems appropriate here.

Summary of Field Investigations

The first known occurrence of the disease was in a herd of dairy cattle near Stillwater, Oklahoma, in November, 1957. The entire producing herd became affected, all cattle developing signs of illness during a 5-day period. A sudden drop in milk production, anorexia, and rapid loss of weight were the first signs noted. This was followed by the development of icterus and by skin lesions characteristic of photosensitization. Lesions developed on the lightly pigmented areas such as the muzzle, around the eyelids, ears, teats, exposed areas of the udder, perineum, neck and upper surfaces of the body.

At the time these cattle became affected they were confined to a small pasture which was practically depleted of native grass and was heavily overgrown with weeds. The tops of the weeds were wilted from effects of the first fall frost which had occurred a few days previously. These factors suggested that the recent "frost burn" might have been a factor in causing development of toxicity.

In early December a similar occurrence of photosensitization was

investigated in a dairy herd near Tahlequah, Oklahoma. The clinical manifestations in affected cattle of this herd were identical to those observed in the Stillwater herd. The majority of the cattle in the herd were affected, including groups on native pasture and groups in lots in which vegetation was absent or scanty. Alfalfa hay had been added to the ration of all except one of the groups a few days before clinical signs of the disease developed. No signs of photosensitization or other indications of the disease occurred in cattle of the latter group, but the majority of animals in those groups receiving the hay were affected. This evidence strongly implicated the hay as the source of the toxic agent. A review of the circumstances involved in the Stillwater herd disclosed that feeding of alfalfa hay was begun only a few days before the disease developed. The hay involved in the two herds originated in different areas of the state, but, in each instance, it was first-cutting alfalfa harvested in the spring of 1957 which had been damaged by flooding from heavy spring rains occurring prior to harvest.

Samples of the suspected hay from the first two outbreaks and from four subsequent outbreaks produced photosensitization in each instance when fed separately to cattle under experimental conditions.

A large number of additional outbreaks of the disease occurred in Oklahoma and surrounding states during 1957-58. Many of these were investigated by members of the Department of Veterinary Pathology of Oklahoma State University and by State and Federal veterinarians. Further information regarding other cases was reported by practicing veterinarians. Results of these investigations disclosed that cattle were the only species affected, that the disease was directly attributable to the feeding of alfalfa hay, and that only first-cutting hay which had been damaged by

frequent heavy rains and flooding was involved. Neither undamaged, good quality, first-cutting hay nor that representing later cuttings was implicated in any of the known outbreaks.

The actual number of herds affected during 1957-58 was not determined. It was estimated that approximately 400 outbreaks occurred in Oklahoma and at least that many more occurred in the adjoining areas of Kansas, Missouri, Arkansas, and Texas. Economic losses were heavy although mortality was quite low. Adult cattle were usually affected more severely and in greater numbers than the younger ones in the herd. Morbidity often reached one-hundred per cent in mature cattle but decreased sharply in young calves. The principle economic effects were due to marked depression of milk production, severe weight loss, some abortions, prolonged unthriftiness, and increased susceptibility to other diseases. Most of the affected animals gradually recovered within a few weeks to a few months.

Efforts were made to reproduce the disease in domestic species other than cattle and in laboratory animals (Monlux et al., 1963). Sheep, guinea pigs, rabbits and horses were fed selected specimens of the hay which had proven to be toxic to cattle. Evidence of photosensitivity was not induced in any of these animals even after extended periods of feeding the toxic hay. Necropsy examinations of animals from these trials did not disclose any gross or microscopic evidence of hepatic damage.

Failure to reproduce the disease in laboratory animals necessitated the use of cattle as the experimental animal in all subsequent investigations.

CHAPTER II

REVIEW OF SELECTED LITERATURE

General Aspects of Photosensitization

Various aspects of photosensitization in domestic animals were presented in the excellent review articles by Clare (1952, 1955, 1956). In these publications the author presented information regarding the mechanisms of development of photosensitization and classified the photosensitivity diseases of animals according to genesis of the different forms which have been observed. Clare's work focused attention on the diverse mechanisms by which photosensitization may develop and emphasized the basic considerations necessary in understanding the essential nature of the various photosensitivity diseases in animals.

Substances which induce hypersensitivity to light are extremely diverse both in chemical structure and in occurrence (Quin, 1933a; Rimington and Quin, 1934; Fourie, 1936; Blum, 1941; Lemberg and Legg, 1949; Brockman, 1957). Some occur naturally in plants. Others are produced in the animal body through both normal and abnormal metabolic processes. Many dyes and stains are also capable of sensitizing animals and *in vitro* biological systems to light. Practically all of these photodynamic agents are colored compounds. Only one instance of photosensitization in animals has been reported in which the photodynamic agent does not absorb in the visible range (Clare, 1947).

Nature and Mechanism of Photodynamic Action

The fundamental aspects and theoretical basis of photochemical and photosensitized reactions, and the etiological relationship of these processes to disease, have been covered comprehensively in a number of monographs (Blum, 1941; 1959; Hollaender, 1955, 1956).

Photosensitized reactions observed in biological systems were first reported by Raab (1900). Tappeiner (1909) designated this phenomenon as "photodynamic action." Substances which are capable of sensitizing biological systems to light are termed "photodynamic agents." The "sensitizers" or photodynamic agents absorb light of specific wavelengths determined by molecular structure of the particular compound. They are thus activated to a higher energy state and catalyze the photochemical reaction without themselves undergoing any permanent change (Blum, 1941; Livingston, 1955).

The property of fluorescence appears to be common to all photodynamic substances (Clare, 1956). Fluorescence, however, is not itself responsible for photodynamic action. It is merely readily observable evidence that the molecules of such substances are capable of excitation by absorption of radiant energy and is, therefore, an associated phenomenon in substances which possess photodynamic activity (Blum, 1941; Livingston, 1955).

The general mechanism of tissue damage by photodynamic action appears to be an oxidation reaction requiring the presence of molecular oxygen (Blum *et al.*, 1935; Blum, 1941). Blum (1941) and Clare (1956) both have suggested that, were it not for long established precident in designating such photosensitized reactions as photodynamic action, a less

general descriptive term such as "photosensitized oxidation" would be preferable.

While there is general agreement that photodynamic action is an oxidation process, details regarding the intermediate steps are not well understood. Several possibilities are discussed by Blum (1959), who concludes that there seems to be no scheme of intimate mechanisms that is not open to question. Clare (1956) states that the different mechanisms suggested by various workers emphasize the possibility that photodynamic action may proceed by various pathways according to the circumstances.

Most of the available evidence indicates that protein is the substrate which undergoes oxidation and that tyrosine, tryptophane, and histidine are the amino acids most susceptible to photosensitized oxidation (Clare, 1956). Many questions concerning the specificity of the reaction, nature of the end products, and how the induced alterations in the protein affects cell structure and function remain unanswered.

Lesions of Photosensitization

External lesions are similar in the various photosensitivity diseases irrespective of the specific photodynamic agent involved. The severity and extent of the lesions, however, may vary considerably depending on such factors as the degree of photosensitivity together with the intensity and duration to light.

When sensitized tissues are exposed to light of appropriate wavelength, progressive degenerative changes are produced and an acute inflammatory reaction rapidly develops. Varying degrees of erythema and edema develop and the more severely affected areas undergo necrosis.

The lesions develop in areas of the body which are not adequately protected from sunlight by hair, wool, and pigment. They involve the epidermis and dermis primarily but may extend into the subcutaneous tissues in the more severe cases.

The gross appearance and distribution of lesions of photosensitization and the clinical signs exhibited by affected animals have been described by various workers (Theiler, 1918; Mathews, 1937; Cunningham et al., 1942; Clare, 1952). The principle microscopic features of the developing skin lesions were described by Mathews (1937) and by Cunningham et al. (1942).

Photosensitization Versus Sunburn

The various aspects of altered response in the skin in association with sunburn and photosensitization and the conditions necessary to elicit the pathological reactions are discussed at length in the treatise by Blum (1941). Additional considerations regarding sunburn are presented by the same author in a more recent publication (Blum, 1955).

The general reaction of the skin in both photosensitization and sunburn appears to be an initial erythema followed by varying degrees of edema and acute inflammatory reaction (Levy, 1929) but the reaction tends to be more severe and extends deeper into the dermis and subcutis in photosensitization (Videbeck, 1931). Despite the similarity of tissue response, there are substantial differences in the fundamental mechanisms causing development of lesions in the two conditions. The salient points of dissimilarity in the latter respects are as follows: (1) development of lesions of photosensitization is dependent on the presence of a photodynamic agent in the exposed tissue; lesions of sunburn develop in normal

skin in the absence of sensitizing agents; (2) signs of photosensitization may be evoked within a few minutes upon exposure to sunlight; signs of sunburn are delayed; (3) pathological effects of photosensitization are elicited by wavelengths of light in the visible and near ultraviolet range usually above 360 millimicrons; sunburn is produced only by ultraviolet light generally below 320 millimicrons; (4) photodynamic action requires molecular oxygen; sunburn is largely independent of oxygen supply in the tissues.

Classification of Photosensitivity Diseases

Cunningham et al. (1942) recognized that the known photosensitivity diseases among animals could be separated into at least three distinct classes according to the manner in which the photosensitization is brought about. Clare (1942) followed the scheme of classification as outlined by Cunningham et al. and grouped the diseases into types according to origin of the photodynamic agent and the mechanism responsible for its accumulation in the peripheral circulation in harmful quantities. The three major classes proposed by Clare are:

Type I. Primary Photosensitivity
Type II. Photosensitivity due to Aberrant Pigment Synthesis
Type III. Hepatogenous Photosensitivity

A fourth category was designated by Clare as "photosensitivity of uncertain etiology." He included in this group those cases of photosensitization which have been reported without sufficient accompanying information to be fitted appropriately into one of the three major types.

Type I. Primary Photosensitivity

In the photosensitivity diseases of Type I, the photosensitization is a primary condition uncomplicated by other pathological processes. All

the lesions are directly attributable to photodynamic action and are not related in any way to disturbed metabolism or function in the body. In naturally occurring cases of primary photosensitivity, the photodynamic agent is produced in plants which are ingested by the animal. The agent is absorbed from the digestive tract and passes freely into the peripheral circulation.

The photosensitivity caused by various species of St. John's wort and by buckwheat are the only well established examples of primary photosensitivities of domestic animals induced by ingestion of plants (Blum, 1941; Clare, 1952). The photodynamic agents in both types of plants are red fluorescent pigments and are of similar chemical structure. Brockman (1957) has identified the pigments from both sources as derivatives of naphthodianthrene. Clare (1952, 1956) suggested that the photosensitivity induced by a few other plants, particularly clovers and related legumes, is possibly of the primary type but this has not been established.

Primary photosensitivity may be induced by the intravenous administration of various fluorescent dyes (Quin, 1933a). A primary photosensitivity in which keratitis is the principle lesion has been reported in various species of animals following phenothiazine therapy (Whitten and Filmer, 1947). The photosensitizing agent was shown to be phenothiazine sulfoxide (Clare et al., 1947) which is formed in the digestive tract from phenothiazine (Clare, 1947).

Type II. Photosensitivity Due to Aberrant Pigment Synthesis

Photosensitivity associated with defective pigment metabolism has been noted only in certain of the porphyric conditions in man and

cattle (Clare, 1956; Watson et al., 1959). Several different forms of porphyria are recognized in man (Eales, 1961) but congenital porphyria is the only type observed in animals under natural conditions. It is a relatively rare disease among both man and animals and is characterized by excessive production of uroporphyrins and coproporphyrins, uroporphyrins usually being produced in highest amounts.

Porphyria in domestic animals has been reported only in cattle (Fourie, 1936, 1939; Jorgensen and With, 1955) and in swine (Clare and Stephens, 1944; Jorgensen, 1959). In cattle and humans the condition is inherited as a recessive trait but appears to be dominant in swine. Clinical cases in cattle and swine can be easily recognized by the pinkish color in the teeth due to deposition of uroporphyrin and coproporphyrin. The bones are similarly pigmented. Both teeth and bones show bright red fluorescence under ultraviolet light.

Porphyria is accompanied by photosensitization in cattle. This feature has not been reported with the disease in swine, although there seems little doubt that the disease is fundamentally similar in the two species. Photosensitivity is observed in the congenital form of human porphyria. There is some question as to whether the cutaneous lesions in the hepatic forms of porphyria are actually lesions of photosensitivity or merely reactions due to the more fragile skin in these patients (Blum, 1941; Clare, 1956).

Type III. Hepatogenous Photosensitivity

This is the most common type of photosensitivity encountered in domestic animals and differs from the previous types in that the development of photosensitization is entirely secondary to and a consequence of

marked disturbance of hepatic function. The photodynamic agent involved has been shown to be phylloerythrin (Rimington and Quin, 1934; Clare, 1944, 1945). This compound is a porphyrin derived from cholorophyll and is formed through the action of micro-organisms in the digestive tract of herbivorous animals (Rothemund and Inman, 1932; Rothemund et al., 1934; Quin et al., 1935). It is absorbed in appreciable quantities from the digestive tract and is excreted in the bile by the liver (Rimington and Quin, 1934; Rothemund, 1935).

The primary hepatic lesions and the associated functional disturbances which result in phylloerythrin retention may occur from a variety of causes but in the naturally occurring photosensitizations of this type the usual causes are hepatotoxic agents associated with plants ingested by herbivorous animals (Clare, 1952; Bourne, 1953). Other less frequent causes of deranged hepatic function which may be accompanied by photosensitization are: obstruction of the biliary tract by inflammatory processes, parasites, and neoplasms, or by surgical ligation of the common bile duct; hepatotoxic action of chemical agents such as phosphorus, carbon tetrachloride, aniline, arsenic, and copper; some infectious diseases which produce severe hepatic damage; and congenital functional defects of the liver.

Specific Hepatogenous Photosensitivity Diseases

"Geeldikkop" (Tribulosis). The disease known in South Africa as "Geeldikkop" is a hepatogenous photosensitization occurring in sheep grazing on the plant, Tribulus terrestris, which becomes toxic during certain periods and stages of growth. The disease is widespread throughout most parts of the Karroo, the Northwest Cape and Southern Free State

of South Africa. A recent review by Brown (1959) gives a comprehensive account of the progress of research on this disease.

"Geeldikkop" was first reported by Hutcheon (1886) who described it as a deeply jaundiced condition of the body with subcutaneous serous effusions especially about the head. He considered the disease to be a primary derangement of the liver but thought it was possibly of malarial origin. Several early attempts to associate the disease with the Tribulus plant were unsuccessful (Dixon, 1899; Hutcheon, 1904; Paine, 1906). Later, in an extensive series of feeding and grazing experiments, Theiler (1918) demonstrated conclusively that "Geeldikkop" was associated with the consumption of the plant Tribulus terrestris.

Theiler (1918) observed that sheep affected by Tribulosis suffered very much in the sun and showed improvement during cloudy weather but he believed the skin lesions to be caused by a toxic agent in the plant. Quin (1928) first suggested that the severe subcutaneous edema and necrosis of skin associated with Tribulosis might possibly be due to photodynamic action. He initiated a series of experiments in which he induced photosensitization by various means and produced identical skin lesions to those of Tribulosis (Quin, 1933a, b, c). Quin (1933c) subsequently produced a condition characterized by icterus and photosensitization similar to that in Tribulosis in sheep and goats by ligation of the common bile duct. In a continuation of this work, Rimington and Quin (1934) showed that phylloerythrin was responsible for the photosensitivity both in the disease induced by ligation of the common bile duct and in natural outbreaks of Tribulosis. These workers concluded that liver dysfunction was the primary disorder in Tribulosis.

The hepatotoxic principle which causes Tribulosis has not been

identified. Brown (1959) suggested that certain sapogenins present in the plant may be partially responsible. Brown and deWet (1962) hypothesized that subclinical selenosis may be involved along with the toxic material present in the Tribulus plant.

Lippia Poisoning. During the investigations concerning Geeldikkop, Quin (1933b) induced a photosensitivity accompanied by icterus in sheep by feeding the plants, Lippia rehmanni (Pears) and Lippia pretoriensis (Pears). The syndrome induced by these plants was very similar to Tribulosis but was milder in degree.

Rimington and Quin (1935) isolated the toxic material from L. rehmanni and gave it the name, "icterogenin." In further investigations, Rimington et al. (1937) reported that icterogenin was composed of three isomeric forms which they designated as icterogenin A, B, and C. All three forms were stated to be physiologically active. Barton and de Mayo (1954) have since found that icterogenin C is a solvated form of A. They were unable to obtain icterogenin B but expressed the belief that icterogenin represents a single substance which they identified as a pentacylic triterpenoid.

Lantana Poisoning. Steyn and Van der Walt (1941) found that the plant, Lantana camara, was the cause of a hepatogenous photosensitization similar to that induced by the Tribulus and Lippia plants. The active hepatotoxic principle was isolated by Louw (1943) and given the name "Lantanin." This material was renamed "Lantadene A" upon subsequent isolation of a similar inactive substance which he named "Lantadene B" (1948). The structure of these compounds was partially elucidated by Louw (1949a, b). Barton et al. (1954) were unable to isolate Lantadene A but established the chemical identity of Lantadene B as a pentacyclic

triterpenoid isomer of rehmannic acid which occurs in the roots of Lippia rehmanni in association with icterogenin.

Lechuguilla and Sacahuiste Poisoning. The photosensitivity syndromes associated with poisoning by Lechuguilla (Agave lechuguilla) and Sacahuiste (Nolina texana) plants are quite similar to each other although the toxic principles in the two plants appear to be of a different nature (Mathews, 1937, 1940). These plants are native to the southwestern region of Texas. Sheep, goats, and cattle may feed on the plants when more palatable vegetation is sparse. Mathews indicated that the photosensitization induced by these plants was consistently accompanied by icterus and reported that lesions of a degenerative nature occurred in both the liver and the kidneys.

The buds, blooms, and ripe fruit of the Sachuista plant all proved to be toxic but attempts to extract the active principle were unsuccessful. The toxic principle in Lechuguilla was found to be present in the leaves, from which it was extractable with both water and alcohol. It possessed the general characteristics of a saponin but was not identified further. Hydrolysis of the alcoholic extract of Lechuguilla caused destruction of the hepato-nephrotoxic principle.

Facial Eczema. Facial eczema is a hepatogenous photosensitivity disease occurring in New Zealand where severe outbreaks have occurred periodically since 1897 (Cunningham et al., 1942). A recent report of an outbreak of the disease in Australia is the only record of its occurrence outside New Zealand (Hore, 1960). The disease occurs predominantly in sheep but cattle may also be affected. The early history and general aspects of the disease are reviewed by Cunningham et al. (1942) and by Clare (1952). An extensive survey of the various ecological relation-

ships to outbreaks of facial eczema was made by Levy and Smallfield (1942). The disease is most prevalent among animals grazing on perennial ryegrass (Lolium perenne). Outbreaks most often occur in late summer and autumn, usually during warm periods when the grass is growing rapidly following rains.

Clare (1944) isolated phylloerythrin from the blood of facial eczema-affected sheep and demonstrated conclusively that this substance was the photosensitizing agent in the disease.

A number of developments, occurring in rapid succession during recent years, have favored rapid progress in facial eczema research. Methods were devised for collecting and storing the toxic grass whereby its toxicity could be maintained over extended periods (Simpson et al., 1957; Clare, 1959b). An empirical chemical procedure was developed for detecting toxicity in grass samples (Perrin, 1959; Sandos et al., 1959). The guinea pig was found to be quite susceptible to the facial eczema toxin and laboratory bio-assay procedures were established using the guinea pig as a test animal (Simpson et al., 1957; Evans et al., 1957; Perrin, 1957). Subsequently this work was extended to the New Zealand white rabbit which proved even more sensitive to the toxic agent than the guinea pig (Clare, 1959a; Dodd, 1960).

Evidence that the facial eczema hepatotoxin is produced by a micro-organism was provided when cultures of the fungus, Sporidesmium bakeri (now Pithomyces chartarum), isolated from toxic pasture samples, were found to induce typical hepatic lesions of the disease when fed to guinea pigs and lambs (Percival and Thornton, 1958; Thornton and Percival, 1959; Percival, 1959a, b). The toxic substance has been obtained in concentrated form both from pasture samples and artificial cultures of the

fungus (White, 1958; Synge and White, 1959, 1960). It occurs in analogous fractions of the extracts and all evidence indicated that the toxin from both sources is identical (White, 1959). The toxic principle has been designated as "sporidesmin" and the elemental composition of the carbon tetrachloride-crystalized material has been tentatively postulated as $C_{19}H_{21}O_6N_3S_2Cl \cdot CCl_4$ (Synge and White, 1959).

The results obtained by a number of workers in bio-assay investigations in which extracts of the toxin from pasture samples and artificial cultures of Pithomyces chartarum (syn. Sporidesmium bakeri), as well as crude fungus material obtained both from pastures and by artificial culture, leave no doubt that the toxic agent in the various preparations and materials has the same biological effect (Murphy and Worker, 1960; Worker and Dodd, 1960; Done et al., 1961; MacKinnon and Te Punga, 1961; Te Punga and MacKinnon, 1961; Mortimer and Taylor, 1962).

It became apparent early in the investigations concerning the relationship of the fungus, Pithomyces chartarum, to facial eczema disease that the toxic material was primarily associated with the spores of the fungus (Percival and Thornton, 1958; Percival, 1959a, b; Thornton, 1959; Thornton and Sinclair, 1960; Sinclair, 1961). A number of subsequent reports have been published concerning spore collection, seasonal variations, distribution of spores on the pasture herbage, and factors affecting the toxicity and germination of spores (Smith, Crawley, and Lees, 1961a, b; 1962a, b; Clare and Gumbley, 1962; Smith and Crawley, 1962; Mitchell et al., 1961). The occurrence, morphology, and taxonomy of P. chartarum has been recently summarized by Dingley (1962).

Panicum Photosensitivity. Steyn (1928) described a photosensitivity disease called "Dikkoor" characterized by generalized icterus

which occurred in sheep in the Orange Free State of South Africa. He believed the disease was probably caused by Panicum maximum since this was the only plant common to all outbreaks but suggested that other Panicum species may have been involved. Rimington and Quin (1937) investigated similar outbreaks of the disease and concluded that it was caused by grazing of Panicum laevifolium and Panicum coloratum a few days following rains. Steyn noted that the grass was heavily infected with smut in all the outbreaks investigated by him but he was unable to reproduce the disease by feeding the infected plants.

Simpson (1946) reported a severe outbreak of photosensitization accompanied by icterus in sheep in New Zealand grazing on Panicum miliaceum. Clare (1952) states that Filmer (1949) found phylloerythrin to be the photodynamic agent in Panicum photosensitivity in New Zealand and that the lesions in the liver, though not as severe, resembled those seen in facial eczema.

Alveld. Alveld is the name applied to a hepatogenous photosensitivity disease which occurs in Norway among lambs grazing on pastures consisting chiefly of a plant belonging to the lily family, Narthecium ossifragum. Ender (1955) was able to reproduce the disease experimentally by oral dosing of sheep with large amounts of saponins extracted from the plant. He also demonstrated that phylloerythrin was the photosensitizing agent in alveld.

Tetradymia Poisoning. The disease of sheep known as "bighead" which occurs in certain western regions of the United States is a photosensitivity apparently of hepatogenous origin. Clawson and Huffman (1935, 1937) noted that "degeneration" of the liver was a frequent finding in sheep after feeding the plants, Tetradymia glabrata and Tetradymia

canescens. Their reports state that a larger number of animals developed photosensitization when given a plentiful supply of green feed. This suggests that phylloerythrin is possibly the photodynamic agent in Tetradymia poisoning.

Ngaio Poisoning. Cunningham and Hopkirk (1945) induced liver damage, icterus, and photosensitization in sheep by feeding leaves of the Ngaio tree, Myoporum laetum, which is native to New Zealand. Phylloerythrin was found in the blood and urine of affected animals.

Bermuda Grass Photosensitivity. Kidder et al. (1951) reported the occurrence in southeastern areas of the United States of photosensitization accompanied by icterus and liver damage in cattle grazing bermuda grass pastures. These workers suggested that phylloerythrin was the photosensitizing agent but it was not clear that actual determinations for phylloerythrin were made in serum from affected animals.

Gibbons (1958) reported that various clover species were often present along with the bermuda grass in outbreaks he observed. He considers that mold growth occurring on the dead plants is responsible for producing the toxin but no direct evidence was presented to support this assumption.

Southdown Photosensitivity. Cunningham et al. (1942) reported the occurrence of a congenital photosensitivity in purebred Southdown sheep in New Zealand. Clare (1944) demonstrated that a disturbance in the excretory mechanisms of the liver was present in affected animals and that phylloerythrin was the photodynamic agent in this disease.

Miscellaneous Hepatogenous Photosensitivity Diseases. Clare (1952) lists several additional plants which have been incriminated as causes of photosensitization probably of the hepatogenous type. These included

Lantana camara, Brachiaria brizantha, Kochia scoparia, Holocalyx glaziovii, Lupinus angustifolius, and the algae, Microcystis flos-aquae and Microcystis toxica.

CHAPTER III

EXPERIMENTAL DESIGN AND PROCEDURES

General Procedure and Tests Employed

Cattle were used as the experimental animal in the present investigation since this was the only species in which definite susceptibility to the toxic material in the hay could be established during prior preliminary studies (Monlux et al., 1963). Photosensitization was produced both by feeding hay of proven toxicity and by ligation of the common bile duct.

The comparative relationships of the disease induced by the two methods weredetermined by clinical observation as well as by liver function studies, alterations in various blood serum constituents, and examination of biopsy and necropsy tissues. The specific examinations employed were: bromsulphalein liver function tests and routine hematologic examinations; determinations of serum bilirubin and phylloerythrin levels; determinations of serum enzyme activity for glutamic-oxalacetic transaminase, glutamic-pyruvic transaminase, and leucine amino-peptidase; and the macroscopic and microscopic examination of tissues obtained by multiple liver biopsies as well as by necropsy of selected animals.

The investigation also encompassed a review of all liver tissue specimens from animals used in prior investigations of the hay-induced disease. This was done to provide more comprehensive information for

reconstructing the pahtogenesis of the hepatic lesions. In addition, normal values for the various serum constituents were established in 60 to 70 healthy adult cattle (Appendices II - IV).

Selection of the battery of examinations performed with each group of experimental animals was based upon results obtained during previous investigations (Glenn, 1961). The test procedures were repeated in consecutive series at regular intervals established for the group.

Experimental Animals and Test Regimen

The cattle used in the present investigation ranged from 1½ to 6 years of age. Most were young adult steers and heifers of mixed breeding. The majority of animals had sufficient white or light colored areas of skin to favor the development of cutaneous lesions of photosensitization.

Photosensitization was induced in three groups of experimental animals by two different methods as follows: (1) by the feeding of toxic hay to one group of 6 animals, and (2) by surgical ligation of the common bile duct in two groups of 2 animals each.

Animals which were fed the toxic hay were placed in dry-lot for 1-2 weeks prior to commencing induction of the disease. During this period they were fed prairie hay ad libitum and a small daily supplement of protein concentrate. The preinduction period provided an opportunity for the animals to become adjusted to dry-lot conditions before feeding of the toxic hay was commenced. A series of 2-4 tests with each procedure was performed on each animal during the preinduction period. Each animal thus served as its own control for comparison with results of later tests.

After completing the initial tests, the animals were transferred to individual box stalls where the toxic hay was supplied as the only source of feed during an 8-day induction period. They were then placed on green bermuda grass pasture. The experimental period during which all tests were continued extended over an additional 20-day postinduction period. The animals which were not sacrificed at this time were further observed clinically until the skin lesions were completely healed.

The toxic hay utilized in this study was obtained from the farm near Stillwater where the first field outbreak of the disease was reported. Its toxicity was established not only in the field outbreak, summarized in the introduction, but also in subsequent feeding trials (Glenn, 1961). The baled hay had been held in barn storage for 4 years at the time of the present feeding trials in 1961. It was originally of poor quality, was coarse and stemmy, and had a brownish color resulting from water damage. During storage, it had become very dry and unpalatable. In order to favor palatability and insure consumption of sufficient quantities by experimental animals, the hay was ground in a hammer mill equipped with a medium coarse screen and then mixed with a small amount of blackstrap molasses, cottonseed meal, and mixed ground grain. Several bales of hay were mixed together at the time of grinding in an effort to provide more uniform distribution of toxic material.

Two of the cattle in which photosensitivity disease was induced by ligating the common bile duct were subjected to a presurgical regimen similar to that of the preinduction period for those which were fed toxic hay. The remaining 2 animals were allowed continued access to green pasture throughout the presurgical period. Ligation of the common bile duct was accomplished by laparotomy in the right flank. The surgery was

performed under local anesthesia achieved by paravertebral nerve block. A single catgut ligature was placed around the common duct just distal to the junction of the hepatic and cystic ducts. All 4 animals were placed on green pasture the day after surgery and further treated in the same manner as those with the hay-induced disease.

Hematologic Procedures

Blood for erythrocyte and leukocyte counts, packed cell volume and hemoglobin determinations was collected at 2- to 4-day intervals varying with the group and stage of the disease. Samples were obtained from the right jugular vein by allowing the blood to flow freely from a 16 gauge bleeding canula into heparinized collecting tubes. Blood films for differential leukocyte count were prepared within 1-2 hours after collection and stained with Wright's stain. Total leukocyte and erythrocyte counts, as well as hemoglobin and packed cell volume determinations, were made within 4 hours after collection of the samples.

Cell counts were performed by standard hemocytometer procedures. Hemoglobin values were measured with a Spencer Hemoglobinometer. Packed cell volume was determined by the microcapillary tube method.

Bromsulphalein Liver Function Test

The Bromsulphalein[1] (BSP) retention test for liver function was performed essentially as outlined by Cornelius and Wheat (1957) and by Cornelius *et al.* (1958) with only minor modifications in technic.

An 8-10 ml blood sample for use as a blank was obtained immediate-

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[1]Bromsulphalein is the trade name of sulfabromphthalein sodium, U.S.P., Hynson, Westcott, and Dunning, Inc., Baltimore, Md.

ly prior to injection of the dye. One gram of BSP in 5% aqueous sterile solution was injected intravenously into the right jugular vein at a uniform rate over a one-minute period. The midpoint of the injection was recorded as zero or mean injection time. Three postinjection blood samples, 8-10 ml each, were collected from the left jugular vein at intervals of 7, 12, and 17 minutes after dye injection to establish the rate of dye disappearance from the blood stream. During periods of marked retention, longer intervals ranging up to 10, 20, and 30 minutes were used.

All blood samples for the BSP test were collected without the use of anticoagulant. After clotting, they were centrifuged to facilitate clot retraction. A 2.0 ml aliquot of serum was transferred to a cuvette and 3.0 ml of 0.1 N sodium hydroxide, plus 1.0 ml of distilled water, were added. The blank was prepared in the same manner from the serum of the preinjection blood sample. Optical density of the samples was read at a wavelength of 565 millimicrons in a Bausch and Lomb Spectronic-20 Colorimeter. Dye concentration corresponding to the optical density readings were obtained from a standard curve prepared by the method of Hepler (1949). Results were expressed as milligrams of dye per 100 ml of serum. Dye concentration for each of the postinjection samples was plotted against the appropriate collection time on semilog graph paper. Since the rate of dye removal from the blood was shown by Mixner and Robertson (1957) to occur in an exponential manner, a straight line connecting the three points was extrapolated to the ordinate intercept at zero time which indicated the initial dye concentration in the blood. The time required for one-half of the injected dye to be removed from

the blood was then estimated directly from the graph and the results were expressed as half-time excretion or clearance rate ($t_{\frac{1}{2}}$) in minutes.

Serum Bilirubin

Blood samples for serum bilirubin determinations were collected in tubes without anticoagulant. The specimens were held under refrigeration until a firm clot was formed. They were then centrifuged to facilitate retraction of the clot and the serum was removed. The serum was kept refrigerated and protected from light to minimize loss of bilirubin activity. Bilirubin determinations were conducted on the same day the samples were collected.

Total and conjugated serum bilirubin levels were determined by Powell's method for the van den Bergh reaction as modified by Sims and Horn (1958). The test samples and respective blanks were appropriately prepared and optical densities measured at 525 millimicrons in a Bausch and Lomb Spectronic-20 Colorimeter. The corresponding bilirubin values in mg per 100 ml of serum were obtained from a previously prepared calibration curve (Sims and Horn, 1958).

Serum Phylloerythrin

Blood samples for phylloerythrin extraction were obtained and the serum was collected as for bilirubin determinations. Approximately 100 ml of the whole blood was obtained per specimen to insure sufficient quantities of serum for two sets of duplicate extractions. Samples were obtained from the 6 animals with the hay-induced disease and 2 of those in which the common bile duct was ligated. The serum was sealed in screw-cap vials, frozen, and stored at -5° C. for approximately 12 months before the

extractions were performed. Since it was not known how much loss of phylloerythrin may occur during storage, the series of serum samples from 2 animals in which the common bile duct was ligated at a later date were divided into 2 fractions. One fraction was extracted the day following collection. The other fraction was frozen and then extracted after 6 months storage at -5° C.

The method of extraction of phylloerythrin was that described by Perrin (1958), which is essentially an ether extraction process. Absorption spectra of the final ether extracts of phylloerythrin were obtained using a Model 14 Cary Recording Spectrophotometer. Absorption measurements were made over the continuous wavelength range of 390-700 millimicrons. The concentration of phylloerythrin was calculated from the absorption coefficient measured at 414 millimicrons. Purity of the sample was checked by determining the ratio between various optical density readings as indicated by Perrin (1958). Concentration of phylloerythrin was expressed in μg/100 ml of blood serum.

Serum Enzyme Assays

Blood samples for all serum enzyme assays were collected and handled in the same manner as those for bilirubin determinations. The reagents used for measuring activity of the enzymes were the standard reagents supplied by the Sigma Chemical Company.[1] The assays were conducted according to the instructions supplied with the reagents and represented modifications of the methods for determining activity of the various enzymes as follows: glutamic-oxalacetic transaminage (GOT)

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[1]Sigma Chemical Company, 3500 DeKalb St., St. Louis 18, Missouri.

and glutamic-pyruvic transaminase (GPT), method of Reitman and Frankel (1957); leucine amino-peptidase (LAP), method of Goldbarg and Rutenburg (1958).

Liver Biopsy Procedures

Liver biopsies were obtained at intervals of 6-8 days during the experimental period. Biopsies were made during the preinduction period for comparison with those taken after the disease was induced.

All biopsy specimens were taken from the right dorsal lobe of the liver. Most were secured by needle puncture technique using a 4-inch Vim-Silverman needle; however, wedge biopsies by laparotomy procedure also were obtained from the animals with the hay-induced disease. Wedge biopsies were obtained from each animal on the 12th day after commencing feeding of the hay, and again on the 32nd day, from 3 animals which were not sacrificed for necropsy examination.

Biopsy specimens were transferred to 10% buffered formalin immediately after removal from the liver, allowed to fix for 24-36 hours, and then embedded in paraffin. Sections were cut at 5 microns thickness and stained with hematoxylin and eosin stain, and with Van Gieson's and Gomori's trichrome stains for connective tissue. Representative specimens were also stained for bilirubin by the method of Hall (1960).

Tissues collected during necropsy for histologic examination were handled in the same manner as the biopsy specimens.

CHAPTER IV

RESULTS

Clinical Observations

There was considerable variation both in the daily and total quantity of hay consumed as well as in the severity of symptoms produced in the 6 animals which were fed the toxic hay. Hay consumption was highest during the first 3 days and progressively declined thereafter. All except 2 animals had ceased eating by the eighth day.[1] The total quantity of hay consumed by each animal ranged from 29-75 pounds and averaged 52.2 pounds per animal. Average daily consumption throughout the 8-day induction period is shown in Figure 1. Complete data concerning hay consumption by each animal is presented in Appendix I.

All animals remained alert throughout the induction period. Weight loss became noticeable by the fifth day and was quite marked by the eighth day in most animals. Water consumption also dropped during this period and the feces tended to become dry and firm. Fever was not apparent in any of the animals. Weight loss averaged approximately 8% during the 8-day induction period, however, it was felt that as much as 30-50% of the weight loss was due to decreased volume of digestive tract

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[1]The day on which feeding of the toxic hay was commenced is used as the reference point for subsequent developments unless otherwise stated.

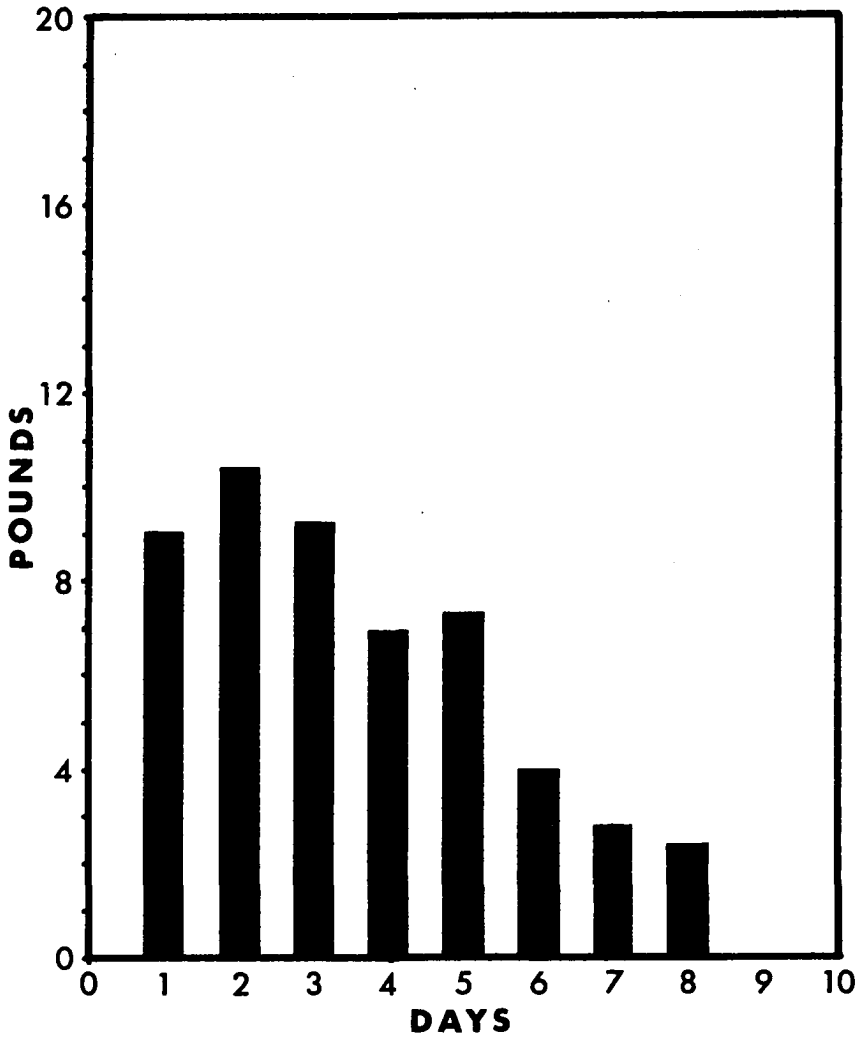

Figure 1. Average daily consumption of toxic hay during the 8-day induction period.

contents. The most severely affected animals continued to lose weight for several days after removal from the toxic hay diet. Those which were less affected began to recoup their weight loss almost immediately and gradually recovered to near their original weights by the end of the 20-day postinduction experimental period.

Clinical icterus and photosensitization first became apparent between the tenth and twelfth days in all 6 animals afflicted with the hay-induced disease. Erythema of the muzzle was usually the most definite indication of developing photosensitization.

The degree and duration of icterus and photosensitivity varied considerably among the different animals but paralleled each other quite closely in each animal. In the very mildly icteric animals, only a slight reddening of the muzzle developed. Both lesions disappeared within 1-3 days. In one animal in which icterus was barely detectable and quite transient, lesions of photosensitivity developed only in the white areas of a narrow strip along the back where the hair had been closely clipped (Figure 2). Lesions were not present in the adjacent unclipped areas of skin or in the pigmented clipped area.

Signs of photosensitivity manifested by the more severely affected animals were photophobia, excessive lacrimation, and frequent licking of the muzzle. As the degree of photosensitivity increased, additional signs noted were shaking of the head, profuse lacrimation, stamping and kicking of the feet, licking at the accessible affected areas of skin, and actively seeking shade. The muzzle and affected areas of skin gradually developed a dark reddish-yellow color as the erythema and icterus became more severe (Figure 3). Upon further exposure to the sun,

the color of the muzzle and affected areas of skin progressed to a dull reddish-brown and began to ooze yellowish serum. Subsequently, the skin became dry, crusty, and hard. The muzzle was especially subject to trauma during this period (Figure 4). Within a few days the affected skin developed fissures, began to curl, and finally sloughed, exposing areas of regeneration and granulation tissue (Figure 5). Complete healing and regrowth of hair occurred in the mildly affected areas within a few weeks. The severely affected areas required longer to heal and regrowth of hair was sparse.

Signs of photosensitization developed on the first day following surgery in 2 of the animals with surgically-induced biliary occlusion but not until the second to fourth day in the other 2 animals. Clinical icterus developed between the second and fourth postsurgical day in all 4 animals. Progressive development of the clinical disease and the lesions of photosensitization were otherwise identical to that in the moderate to severely affected animals with the hay-induced disease (Figures 6 and 7).

Two of the animals with surgically-induced biliary occlusion developed the disease to a marked degree but the remaining 2 animals were only moderately affected. An explanation for this difference was disclosed at necropsy when the moderately affected animals were found to have incomplete biliary occlusion. Complete occlusion of the common bile duct had been accomplished in the more severely affected animals.

Serum Bilirubin

All animals in the three experimental groups developed significant elevations in the serum bilirubin levels. There was considerable

Figure 2. Mild photosensitization of skin 3 days after initial erythema developed. The lesion is very superficial and developed only in the non-pigmented skin along the clipped streak. Healing occurred rapidly. Hay-induced disease.

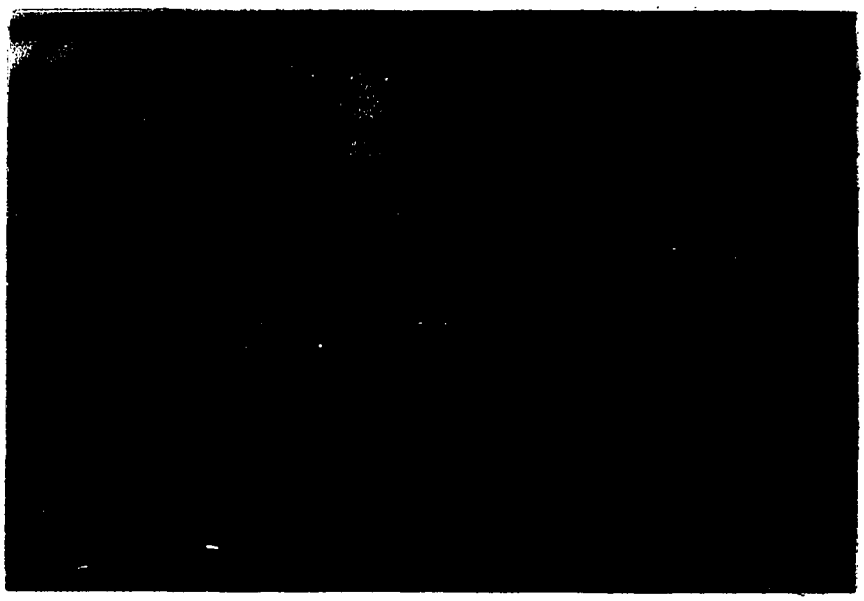

Figure 3. Early muzzle lesions 2 days after initial erythema and icterus developed. This animal subsequently developed severe skin lesions. Hay-induced disease.

Figure 4. Severe muzzle lesions 20 days after initial lesions developed. Patches of the necrotic surface layer have peeled away exposing the underlying granulation tissue. Hay-induced disease.

Figure 5. Advanced skin lesions in a moderately affected animal 30 days after initial lesions developed. Large patches of skin are sloughing. Only the white areas were affected. Hay-induced disease.

Figure 6. Advanced skin lesions in a severely affected animal 30 days after initial lesions developed following ligation of the common bile duct. The lesions are more extensive than those in Figure 5. Only the white areas are involved.

Figure 7. Lesions of the leg in the same animal as in Figure 6. Similar lesions developed in the white areas over the entire body except the more protected underneath areas.

difference in the peak levels reached in the different animals, both among those with the hay-induced disease and those in which the disease was produced by bile duct occlusion. Elevations in the serum bilirubin involved both the unconjugated and conjugated fractions, the latter showing a greater percentage of elevation.

In animals with the hay-induced disease, the initial rise in serum bilirubin began by the sixth day of the induction period and continued to rise very rapidly, reaching peak levels within 3-7 days after the initial rise began. The levels then decreased rapidly to the general range of preinduction values. The peak levels of total bilirubin ranged from 3.2-11.6 mg/100 ml of serum. Those animals which developed the higher serum bilirubin levels required a longer period to reach the peak elevation and required a correspondingly longer time to return to normal. Serum bilirubin values for each of the 6 animals with the hay-induced disease are presented in Figure 8.

Serum bilirubin levels in animals with surgically-induced biliary occlusion rose very rapidly following ligation of the common bile duct. Each of the 4 animals had total bilirubin levels above 2 mg/100 ml of serum by the end of the first postsurgical day. Bilirubin levels in the serum of the 2 animals in which complete biliary occlusion was accomplished rose quite rapidly during the first 6-8 days then began leveling out. The peak levels reached were 10.1 and 20.2 mg/100 ml of serum. The 2 animals with incomplete occlusion of the bile passages developed peak levels of 5.3 and 6.2 mg/100 ml serum during the first 4-5 days then declined slightly but remained elevated considerably above normal. In one of the latter animals the serum bilirubin began a further slow eleva-

tion after several days. The major portion of the increased serum bilirubin, as in the animals with the hay-induced disease, was represented by the conjugated fraction. Serum bilirubin values for each of the 4 animals with the surgically-induced disease are presented in Figure 9.

Serum Phylloerythrin

Phylloerythrin was not detected in the serum of any of the experimental animals during the preinduction and presurgical periods. It first appeared in the serum of all animals as the disease began to develop and disappeared as recovery occurred.

In animals with the hay-induced disease, phylloerythrin did not appear in the serum until the eighth to tenth days after beginning the feeding of toxic hay. It then rose sharply, reaching peak levels on the tenth to twelfth days, and subsequently declined. There was considerable variation in the peak levels reached in the different animals. The range varied from 7-107 μg/100 ml of serum. The general pattern of rise and fall in serum phylloerythrin was similar to that of the bilirubin levels. The comparative serum levels of phylloerythrin and bilirubin in each animal with the hay-induced disease are presented in Figure 8.

In the animals with surgically-induced biliary occlusion, the first appearance of phylloerythrin in the serum and its subsequent rapid elevation to peak levels followed the serum bilirubin pattern in much the same manner as in the animals with the hay-induced disease. Phylloerythrin was detected in the serum of all 4 animals within the first 2 days after ligation of the common bile duct. The levels rose sharply and reached their peak levels by the sixth to the twelfth postsurgical day. After reaching peak levels, the serum phylloerythrin began to decline fairly

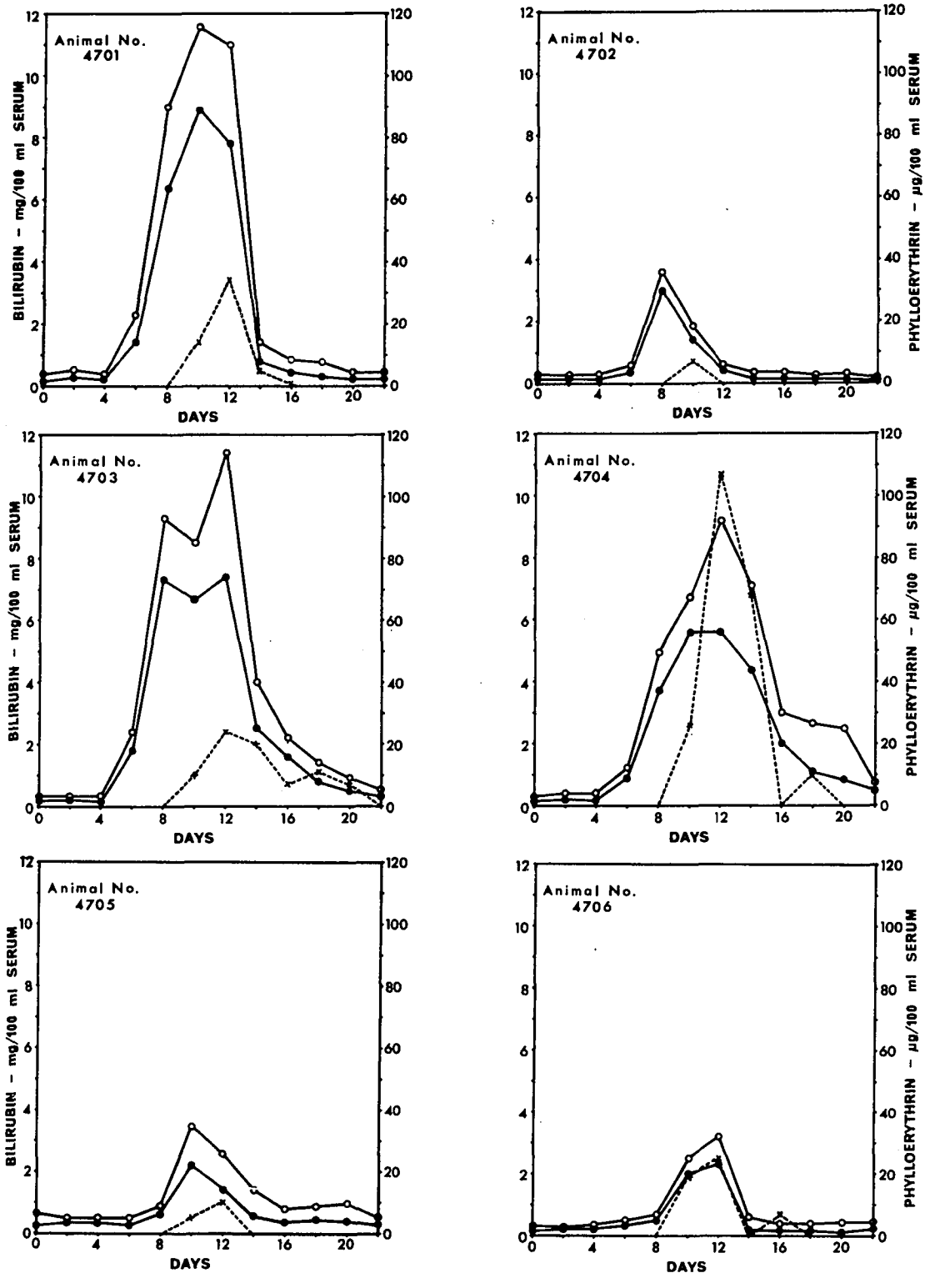

Figure 8. Serum levels of bilirubin and phylleorythrin in the 6 animals which were fed the toxic hay. The 8-day feeding period (induction period) began on 0-day.

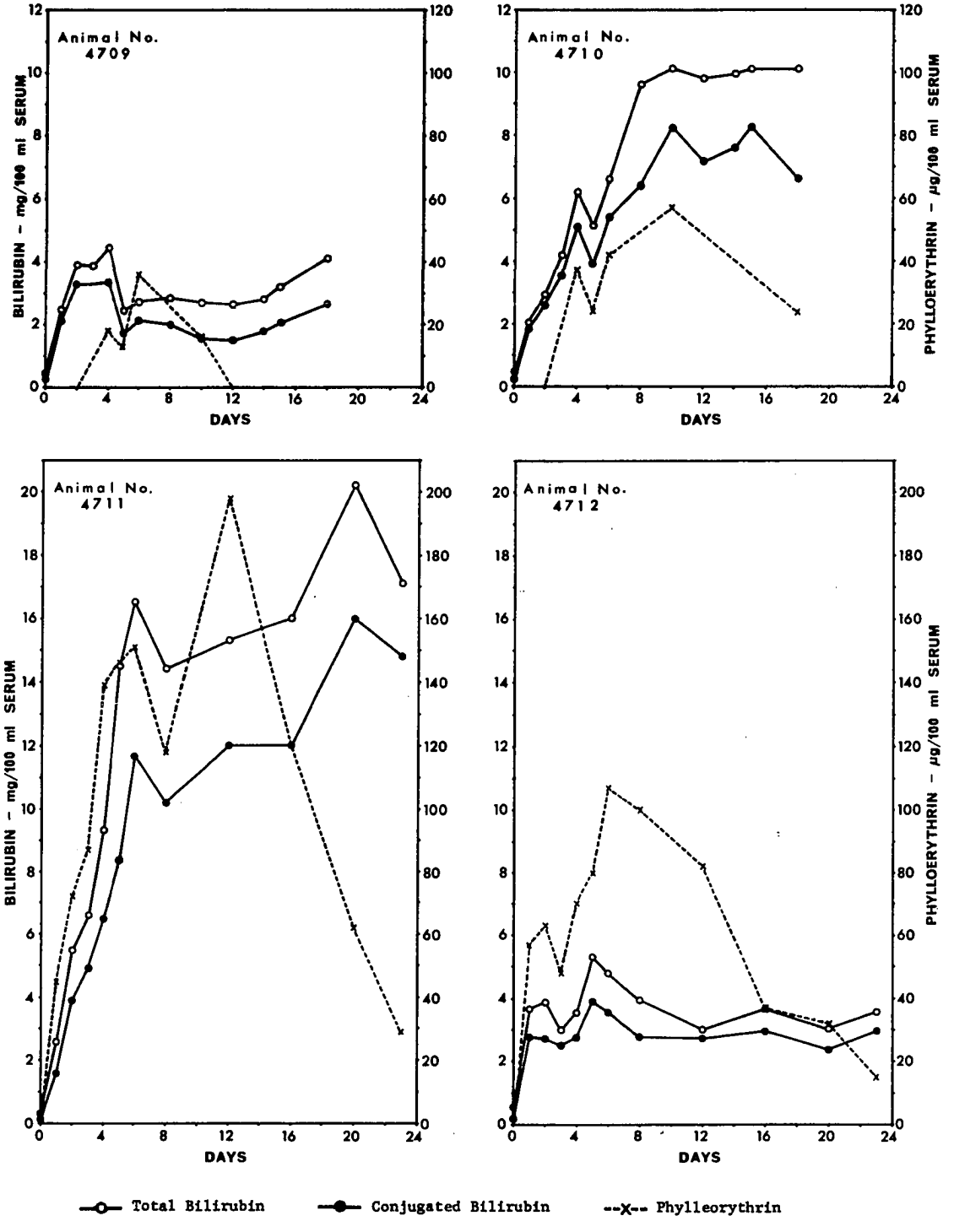

Figure 9. Serum levels of bilirubin and phylleorythrin in the 4 animals in which the common bile duct was ligated. The ligations were performed at 0-day.

rapidly and disappeared entirely from the serum in one of the animals with incomplete biliary occlusion. The comparative serum levels of phylloerythrin and bilirubin in each animal with the surgically-induced disease are presented in Figure 9.

The phylloerythrin determinations on fresh and stored serum indicated that considerable loss occurred during storage. The amount lost during the 6-month storage period ranged from 17-45% in the different serum samples. The average loss in all samples was 31% in one animal and 33% in the other (Table I).

Solutions of phylloerythrin as it appears in the final ether extract from the serum are shown in Figure 10. The high degree of fluorescence which this material exhibits is illustrated in Figure 11.

Bromsulphalein Liver Function Tests

Bromsulphalein (BSP) tests for liver function were conducted at 4-day intervals in each of the animals with the hay-induced disease and at 2-4 day intervals in 2 animals with surgically-induced biliary obstruction. Marked elevations in the half-time clearance ($t_{\frac{1}{2}}$) values occurred in all animals but the degree and duration of elevated values varied widely.

In animals with the hay-induced disease, the initial rise in BSP retention occurred between the fourth and eighth days after feeding of the toxic hay was commenced. The $t_{\frac{1}{2}}$ values rose sharply following the initial elevations with highest BSP retention occurring between the eighth and twelfth days. The peak $t_{\frac{1}{2}}$ values measured ranged from 48.5-103 minutes as compared to the normal values of 4.7 ($\pm$ 0.83) minutes. BSP retention tended to decrease rapidly following the peaks. The

TABLE I

LOSS OF SERUM PHYLLOERYTHRIN DURING STORAGE

Sample Date	Animal No. 4711			Animal No. 4712		
	Serum Phylloerythrin µg/100 ml serum		Percent loss during storage	Serum Phylloerythrin µg/100 ml serum		Percent loss during storage
	Fresh serum	Stored serum		Fresh serum	Stored serum	
8/1/62	45	---	--	57	34	40
8/2/62	72	47	35	63	47	25
8/3/62	87	51	42	48	40	17
8/4/62	139	98	29	70	52	26
8/5/62	146	115	20	80	63	21
8/6/62	151	98	35	107	67	37
8/8/62	118	78	34	100	73	27
8/12/62	198	103	48	81	51	37
8/16/62	120	71	41	37	22	40
8/20/62	62	34	45	31	19	39
	Mean loss --		33%	Mean loss --		31%

Figure 10. Solutions of phylloerythrin in ether. The specimen on the right is a composite sample from several extractions which have been further concentrated. The two specimens on the left each represent the final extract from 10 ml serum samples.

Figure 11. Fluorescence of phylloerythrin solution in a beam of ultraviolet light. The specimen is the same as that shown on the left in Figure 10.

duration of elevated $t_{\frac{1}{2}}$ values above normal in each animal was in direct proportion to the degree of highest values reached. The degree of BSP retention in each animal throughout the development and recovery course of the disease is presented in Figure 12.

BSP clearance tests were performed only in the animals with surgically-induced biliary occlusion. In these 2 animals, BSP retention began to rise very rapidly following ligation of the common bile duct. The half-time clearance values by the second postsurgical day were well above the minimum peak level which was encountered among the animals with the hay-induced disease. In both animals an initial retention peak was reached by the fourth day. This was followed by a second peak on the fifteenth day. The highest $t_{\frac{1}{2}}$ values measured for the 2 animals were 120 and 99 minutes. High $t_{\frac{1}{2}}$ values persisted in both animals until they were sacrificed on the eighteenth day. Results of the BSP tests for these 2 animals are presented in Figure 13.

Normal $t_{\frac{1}{2}}$ BSP clearance values measured in 62 clinically healthy cattle of various ages are presented in Appendix III.

Serum Enzymes

Glutamic-oxalacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and leucine amino-peptidase (LAP) serum enzyme assays were performed in the same groups of animals as were the BSP tests. The activity of each of these enzymes was checked in serum samples at intervals of 1-2 days throughout the experimental period.

The GPT and LAP activity in the serum remained within normal limits in all animals regardless of the method of inducing the disease. Elevations in GOT activity of the serum occurred in all animals but reached

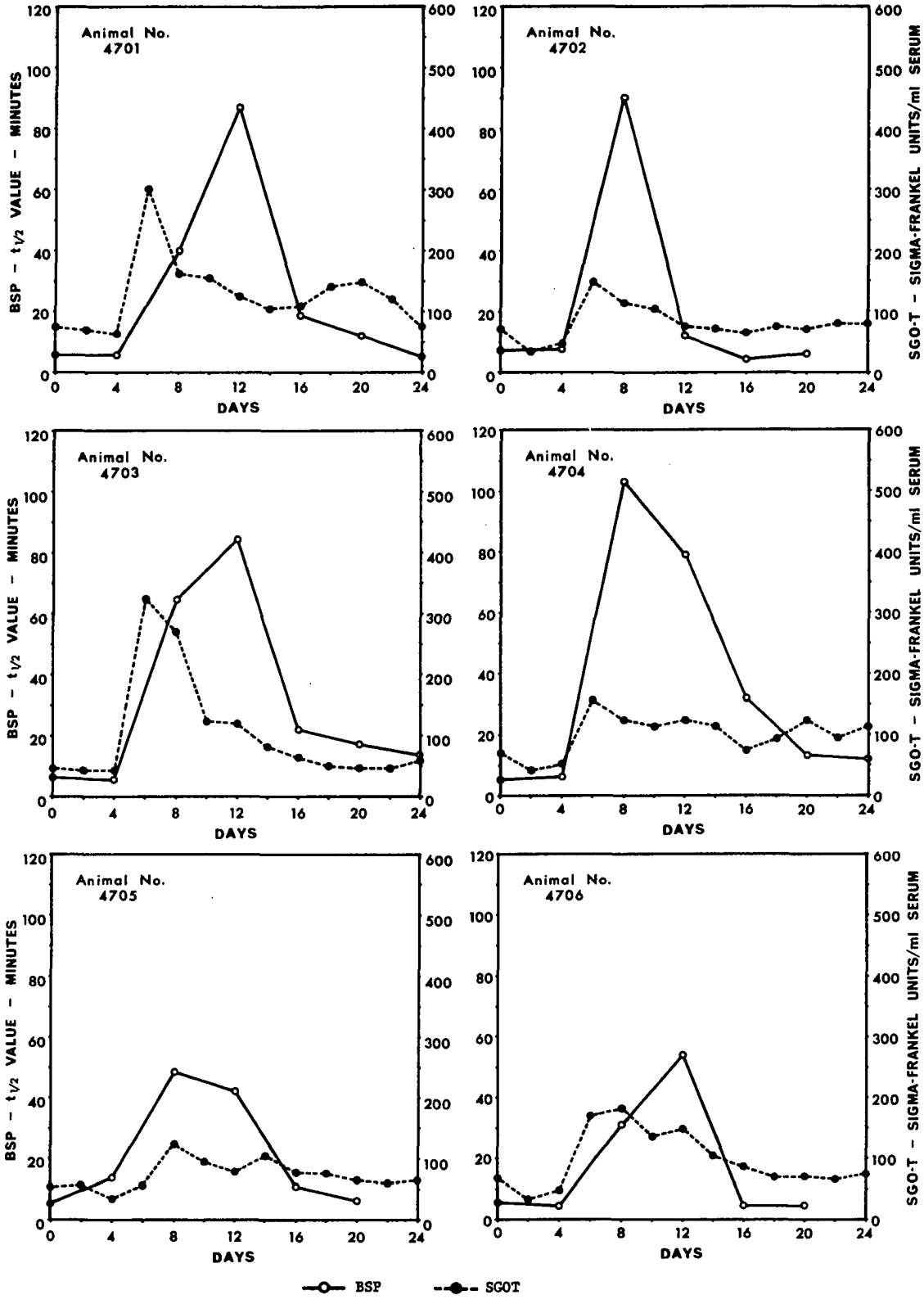

Figure 12. Bromsulphalein (BSP) clearance and serum glutamic-oxalacetic transaminase (SGOT) activity in the 6 animals which were fed the toxic hay. The 8-day feeding period began at 0-day.

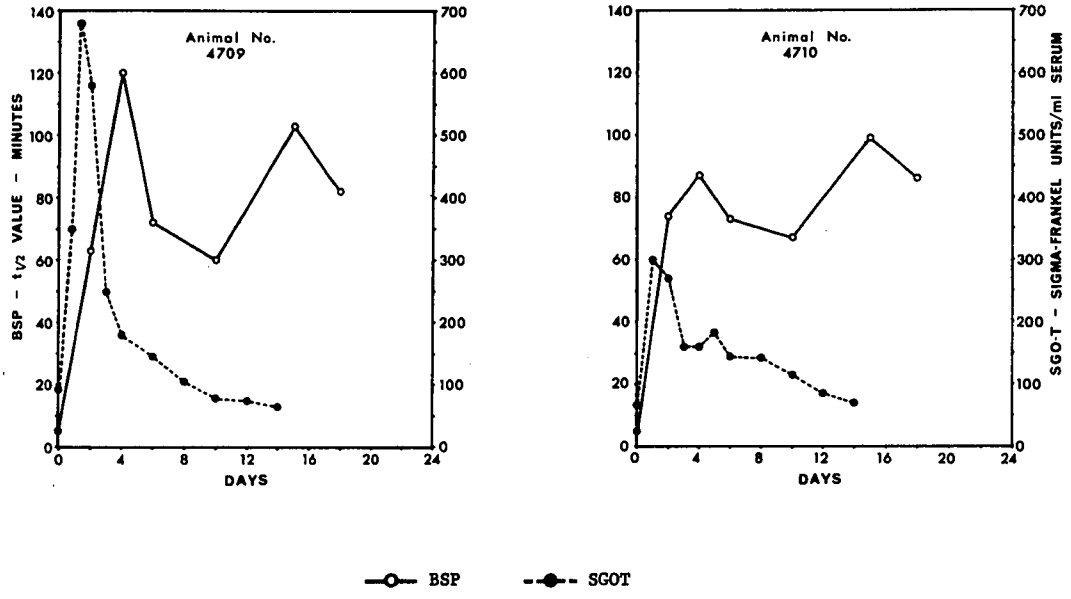

Figure 13. Bromsulphalein (BSP) clearance and serum glutamic-oxalacetic transaminase (SGOT) activity in 2 of the animals in which the common bile duct was ligated. The ligations were performed at 0-day.

much higher levels in those with surgically induced biliary obstruction. Peak elevations occurred within the first 2 days following ligation of the common bile duct and within 2 days after the initial rise began in most animals with the hay-induced disease. The GOT activity began to rise at about the same time that the rise in BSP retention began but it tended to rise more sharply, reached a peak at a slightly earlier time, and declined more precipitously than did the BSP half-time clearance (Figures 12 and 13).

Values for GOT, GPT, and in the LAP activity in the serum measured in 64 healthy adult cattle are presented in Appendix IV.

Hepatic Lesions

The progressive development of hepatic lesions associated with the hay-induced disease was studied in biopsy and necropsy material from a total of 22 animals. Tissue specimens examined during this study included those obtained from 16 animals used in prior investigations as well as the 6 animals in the present group which were fed the toxic hay. The specimens included periodic liver biopsies from 20 animals as well as necropsy material from 7 animals sacrificed at different stages during the disease course. No significant lesions were observed in any of the internal organs other than the liver; however, icteric discoloration of the tissues was present in animals sacrificed during stages of bile retention.

Very little grossly visible change occurred in the liver specimens from animals which developed only mild degrees of photosensitization or in those in which the course of the clinical disease was very short. A distinct yellowish-brown color was noted in liver specimens as bile

retention became more pronounced. Additional macroscopic lesions were seen in the more severely affected animals.

The earliest grossly detectable alterations in the liver of severely affected animals was a slight thickening of the portal spaces, followed by yellowish discoloration of the parenchyma. These changes resulted in a mild accentuation of the lobular architecture. The early lesions were more easily observed on cut surface. The liver developed a more intense yellowish discoloration as the disease progressed.

Further changes in gross appearance of the liver occurred in direct proportion to the duration of disturbed hepatic function. In the animals in which abnormal hepatic function, icterus, and photosensitivity were mild or of short duration, no further changes were evident and the appearance of the liver rapidly returned to normal. In animals which exhibited clinical manifestations of severe photosensitivity over an extended period, the portal tracts were more accentuated, the parenchyma developed an intense orange-yellow hue, and the liver cut with slightly increased resistance. The latter change did not develop to the point of being a pronounced feature in any of the animals.

In 2 of the most severely affected animals there was sufficient thickening of the portal tracts and proliferation of the interlobular connective tissue to cause a slight outlining of many lobules. Bile discoloration was intense and had an irregular distribution pattern which imparted a somewhat mottled effect to the cut surface of the liver. These gross changes appeared to be greatest at about 6 weeks duration. At the time of necropsy examination, approximately 4 months after the initial lesions developed, bile staining of the liver had

almost completely disappeared and the interlobular connective tissue was less noticeable.

Histologic examination of the liver specimens disclosed that the essential lesion was an acutely developing cholangitis and pericholangitis leading to rapid occlusion of the lumen. The small and intermediate interlobular bile ducts were chiefly affected. All animals exhibited some degree of involvement but there was marked variation in severity and duration of the bile duct lesions in the different animals.

The mildest recognizable lesion was a distinct hypertrophy and hyperplasia of the biliary epithelium, most noticeable in the ductules and small interlobular ducts. The epithelial cells lining these small bile channels increased in size, became more basophilic, and mitotic figures were numerous. Crowding of the cells which resulted from these changes was sufficient to completely obliterate the lumen of large numbers of the ductules and small interlobular ducts. No further lesions developed in animals in which only mild, transient clinical signs of photosensitivity occurred. Lumenal patency of the affected bile passages was re-established within a very short period. No residual morphologic changes were noted in subsequent biopsy specimens taken a few days later.

In animals affected to a somewhat greater degree, a mild periductal edema accompanied the hypertrophy and hyperplasia of the ductal epithelium. In addition, small numbers of lymphocytes, macrophages, a few eosinophils, and occasional neutrophils invaded the immediate periductal area (Figure 14). The periductal changes became more pronounced with increasing severity of the disease. Periductal edema was usually greatest before the epithelial hyperplasia and inflammatory cell response

became pronounced (Figure 15). The initial edema was followed immediately by more pronounced hypertrophy and hyperplasia of the ductal epithelium. The latter changes were accompanied by an influx of various types of inflammatory cells into the periductal zone (Figure 16). The periductal edema then regressed rapidly but hyperplasia of the epithelium tended to become more marked (Figure 17). The lesions then underwent a rapid regression. All evidence of active inflammation disappeared within 10-20 days after onset of the lesions.

Residual morphologic changes in the portal tracts in the moderately affected animals were very slight. Only a mild increase in fibrous tissue in scattered portal spaces was observed after a few weeks. Significant morphologic changes were not noted in the hepatic parenchymal cells at any stage of the disease in those animals in which only mild or moderate biliary tract lesions developed.

Hepatic lesions in the more severely affected animals, though of similar character to those in mildly to moderately affected animals, were much more pronounced. The severity of the ductal lesions, the extent of ductal involvement, duration of the lesions, and the degree of residual effect in the liver was directly proportional to the severity and duration of the clinical disease.

In severely diseased animals, ducts of all sizes were affected but the small and intermediate interlobular ducts were involved to the greatest extent and degree. A marked periductal edema developed rapidly and was accompanied by varying degrees of damage to the bile duct epithelium (Figure 18). Complete disruption of the epithelial lining occurred in some ducts. As the periductal edema reached maximum development,

inflammatory cells infiltrated the peripheral edematous area. These cells were of the same type and relative proportions as noted in the less severely affected ducts but were much more numerous. Many of the hepatic parenchymal cells immediately adjacent to the affected ducts developed vacuolar changes and a "feathery" degeneration of the cytoplasm (Figure 19). As the inflammatory cell influx occurred, hypertrophy and proliferative activity began to occur in the periductal connective tissue and in the ductal epithelium (Figure 20). The proliferative activity became quite pronounced as the periductal edema began to disappear (Figure 21). Signs of active inflammation gradually subsided but regenerative activity of the ductal epithelium and proliferation of the periductal connective tissue continued for several days. The lesions were completely healed within 4-6 weeks after their initial onset.

Residual lesions in the liver consisted of variable degrees of ductal proliferation and fibrosis of the portal tracts. These changes developed in proportion to the magnitude of the initial lesions as well as to the duration of the inflammatory process (Figures 22 and 23). The more severe lesion in Figure 23 was taken from an animal used in toxicity tests of three different specimens of hay during preliminary studies; however, the clinical observations indicated that the effect of the last two feedings were not severe.

Even though bile retention was severe, as indicated by serum bilirubin levels and icterus, there was remarkably little bile pigmentation in the hepatic parenchymal cells. Whenever bile pigment was observed in the parenchymal cells in the histologic sections, it was slight and was detected only in cells adjacent to affected portal tracts. Accumulation

of bile in the cannaliculi was not observed in any of the animals. In one animal in which the disease was particularly severe and prolonged, scattered lakes of inspissated bile were observed in a wedge biopsy specimen obtained 8 weeks after development of the initial bile duct lesions (Figures 24 and 25). These were not detected in the preceding punch biopsy specimens. The location of these bile lakes at the periphery of the lobules adjacent to scarred portal tracts indicated that they resulted from rupture of damaged bile ductules.

Figure 14. Small interlobular bile duct with minimal periductal reaction. Hypertrophy and hyperplasia of the epithelial cells have resulted in complete obliteration of the duct lumen. H&E stain. x320.

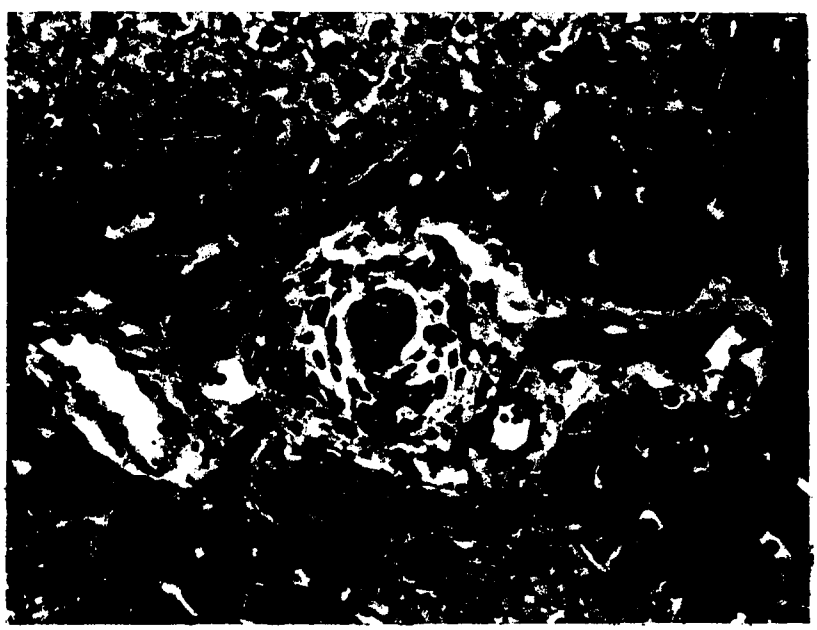

Figure 15. Small interlobular duct with associated periductal edema. Epithelial hyperplasia and inflammatory cell infiltration are minimal. The duct lumen is obliterated. H&E stain. x320.

Figure 16. Ductal and periductal changes at intermediate stage of development. Inflammatory cells have invaded the edematous periductal area and the ductal epithelium is developing more marked hypertrophy and hyperplasia. H&E stain. x320.

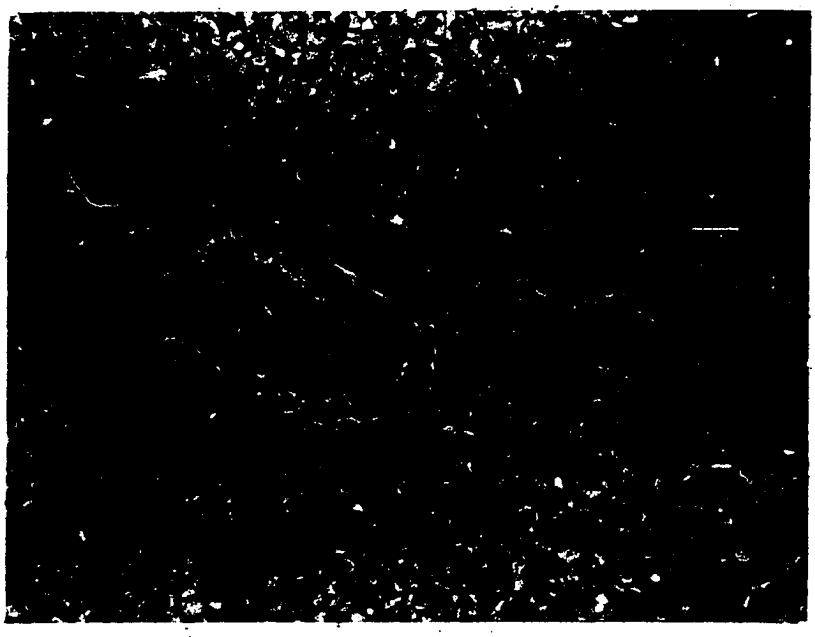

Figure 17. Marked epithelial hyperplasia in a small interlobular duct. Periductal edema has regressed but the inflammatory cell reaction is still present. H&E stain. x250.

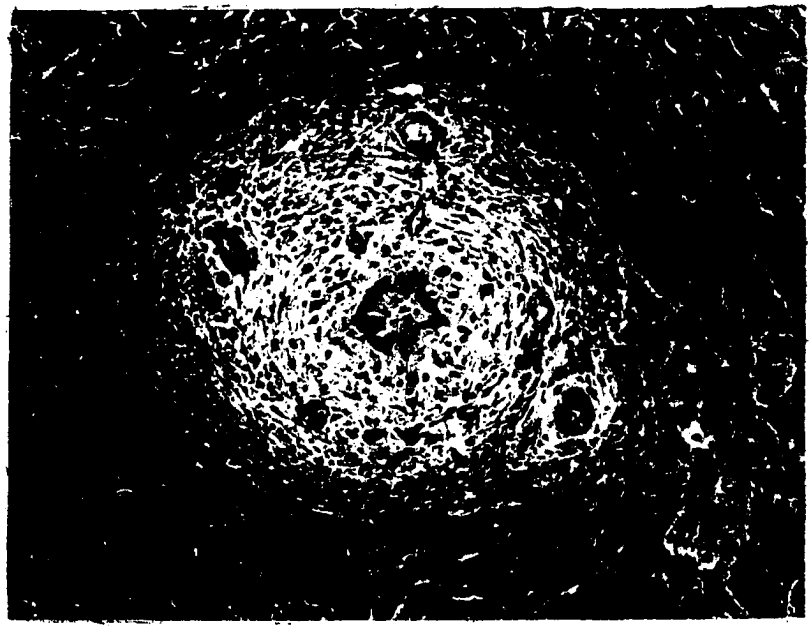

Figure 18. Marked periductal edema involving a duct of intermediate size. Patches of the ductal epithelium exhibit varying degrees of degeneration and necrosis. The surrounding parenchyma is obviously distorted and compressed by the expanded portal tract. H&E stain. x125.

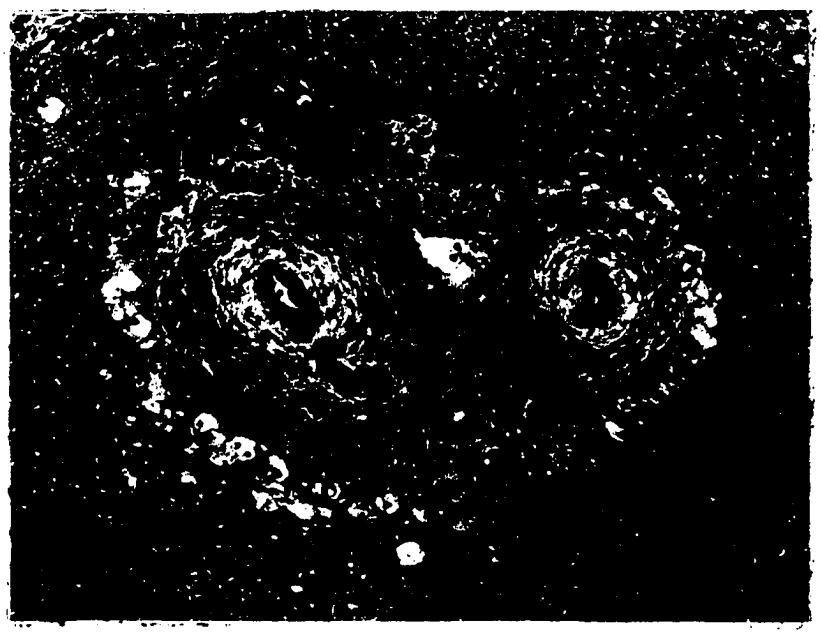

Figure 19. Degenerative changes in parenchymal cells immediately surrounding involved ducts. Hypertrophy and hyperplasia is beginning to occur in the periductal connective tissue and in the ductal epithelium. H&E stain. x125.

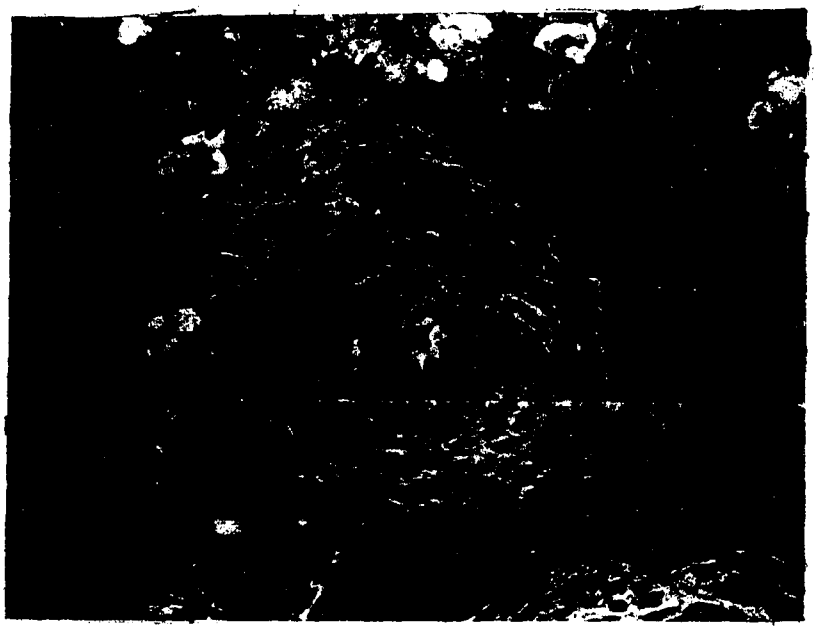

Figure 20. Severely damaged duct with beginning proliferative activity in the periductal connective tissue and biliary epithelium. The epithelial lining of the duct has been completely disrupted but some intact cells remain. H&E stain. x250.

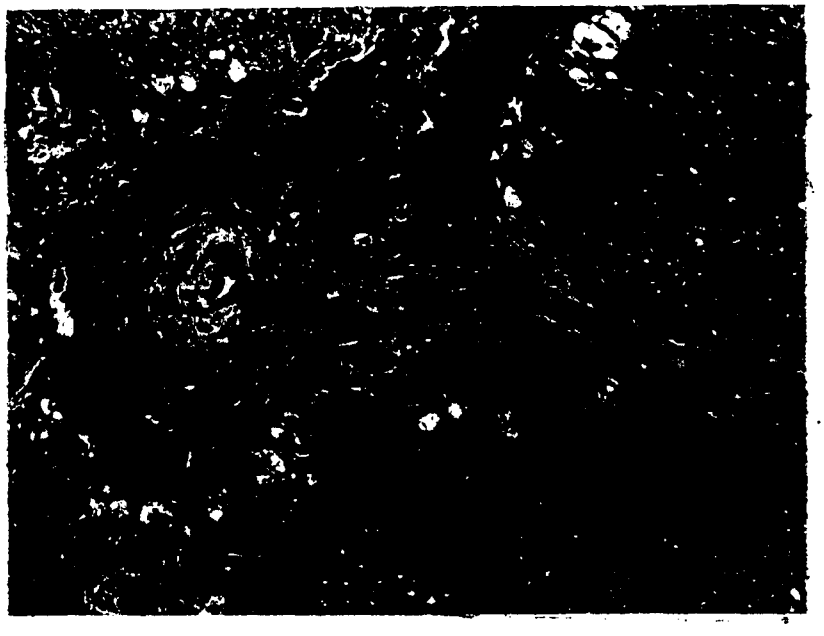

Figure 21. Pronounced epithelial hyperplasia in an intermediate sized duct during the early repairative stage. Several mitotic figures were seen in high power view. H&E stain. x125.

Figure 22. Moderate bile duct proliferation and scarring. Biopsy specimen obtained from a severely affected animal five weeks after initial lesions developed. H&E stain. x100.

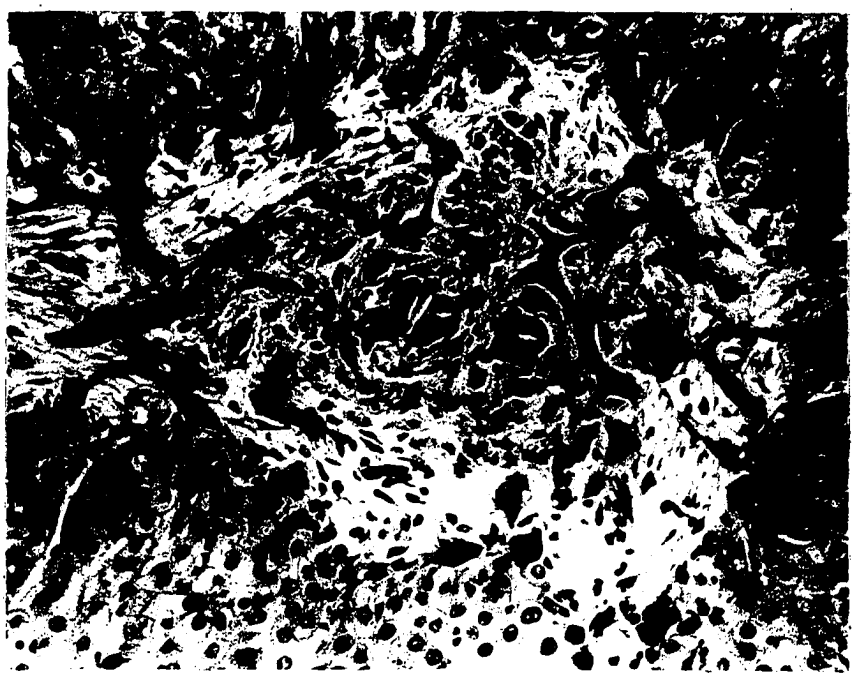

Figure 23. Marked bile duct proliferation and scarring. This animal was fed toxic hay on three separate occasions. H&E stain. x250.

Figure 24. Bile lakes in a biopsy specimen obtained eight weeks after development of initial lesions in a severely affected animal. H&E stain. x100.

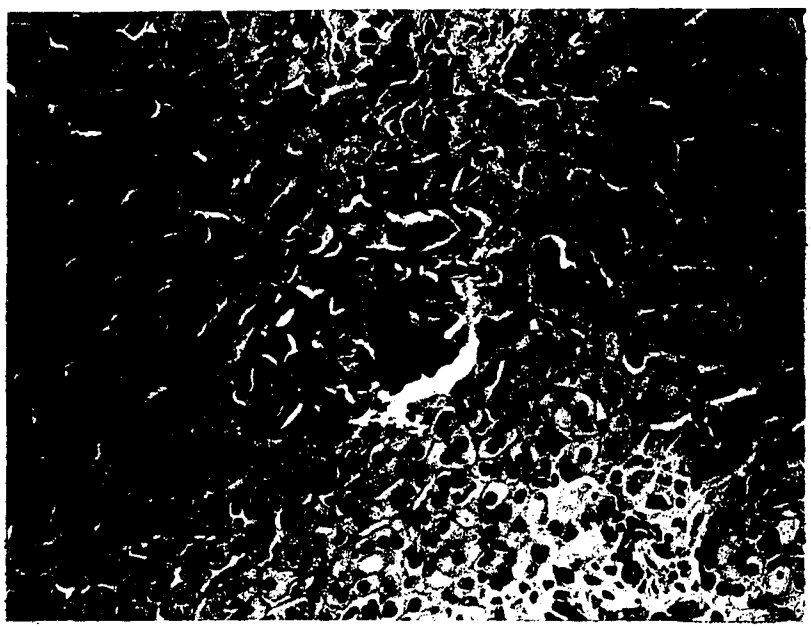

Figure 25. High power view of large bile lake in Figure 22. H&E stain. x250.

CHAPTER V

DISCUSSION

The clinical picture, as well as alterations in the various serum constituents and liver function tests, followed a similar pattern in all animals irrespective of the method by which the disease was induced. The degree of response in all cases appeared to be directly proportional to the degree of biliary occlusion both in the animals with the hay-induced disease and in those with surgical occlusion of the common bile duct.

Each of the animals among the group which were fed the toxic hay consumed sufficient quantity of the hay to cause the disease to develop; however, there was no direct relationship between the total amount of hay consumed and the degree of severity of the disease which developed. The two animals which consumed the largest quantity of hay developed a milder form of the disease than some of those which consumed lesser amounts. Since variation was noted in this respect during previous investigations, it would appear that there is considerable difference in individual susceptibility to the toxic agent involved (Glenn, 1961). However, uneven distribution of the toxin must also be considered as a possible factor. Even though the hay was mixed at the time of grinding, the mixing may not have been sufficient to insure a uniform distribution of the toxin in all portions of the hay.

Considerable evidence was accumulated during the present studies

to indicate that phylloerythrin was the photodynamic agent in the animals with the hay-induced disease as well as in those with surgically induced biliary occlusion.

Clinical photosensitization occurred in all animals, varying only in intensity and duration. It was generally more severe in animals with surgically occluded bile ducts. There was a direct relationship between the development of photosensitization and the presence of phylloerythrin in the serum regardless of the method by which the photosensitivity was induced. Phylloerythrin was present in the serum of all animals throughout the period in which they manifested clinical signs of photosensitivity. It was not detected in any animal before clinical photosensitivity became evident nor was it detectable after photosensitivity disappeared.

There was also a very close correlation between the serum levels of phylloerythrin and the intensity of photosensitization in each animal. Those animals in which the lower levels of phylloerythrin occurred developed only mild degrees of photosensitivity; those with high serum phylloerythrin levels developed signs of intense photosensitivity.

Rimington and Quin (1934) and Clare (1944) have shown that phylloerythrin is a potent photodynamic agent. Rothemund and Inman (1932) and Rothemund et al. (1934) showed that phylloerythrin was a product of chlorophyll degradation produced by the action of microorganisms in the digestive tract. Rimington and Quin (1934) showed that part of the phylloerythrin was absorbed from the digestive tract and normally excreted in the bile. They further demonstrated that phylloerythrinemia and photosensitization developed concurrently in sheep following ligation of the common bile duct.

In the present studies there was a direct relationship between the development of phylloerythrinemia and the amount of chlorophyll in the diet. Among the animals with surgically occluded bile ducts, 2 were allowed access to green pasture during the presurgical as well as the postsurgical period while the other 2 were maintained for several days prior to surgery on a dry hay diet which was relatively low in chlorophyll. The former 2 animals developed a phylloerythrinemia concurrently with the postsurgical bilirubinemia while phylloerythrin was not detected in the serum of those on the low chlorophyll diet until 2-3 days after they were placed on green pasture even though bilirubinemia developed during the first postsurgical day. The same relationship was also noted in animals with the hay-induced disease. Phylloerythrinemia did not occur in the latter animals until 1-3 days after they were placed on green pasture even though most had developed a bilirubinemia much earlier. These observations are in agreement with those of Rimington and Quin (1934). They found that the development of phylloerythrinemia lagged 1-3 days behind the introduction of green feed to the diet of sheep with surgical occlusion of the common bile duct.

In view of the above findings and their excellent agreement with the observations of other investigators, there seems to be no doubt that phylloerythrin was the photodynamic agent in the animals with the hay-induced disease as well as those with surgical occlusion of the common bile duct.

The obstructive nature of the bile duct lesions in animals with the hay-induced disease was borne out by: (1) the histological indications that the hepatic lesions resulted in partial to complete closure

of the lumen of many of the interlobular bile channels, (2) the increased levels of serum bilirubin in the absence of excessive erythrocyte destruction, (3) the greater percentage of serum bilirubin being represented by the bilirubin-glucuronide fraction, (4) the elevated BSP retention in the absence of evidence of morphologic damage to the hepatic parenchyma, and (5) by the close similarity between the various aspects of the hay-induced disease and the disease produced by ligation of the common bile duct.

There was very good correlation between the serum bilirubin levels and BSP retention among the different animals. The beginning elevations, the peak elevations, and subsequent decline in the respective values occurred at corresponding times regardless of the method of inducing the disease. BSP retention tended to be a more sensitive indicator of the milder degrees of involvement than did the serum bilirubin. The similarity of the changes in serum bilirubin levels and BSP retention which occurred in animals with the hay-induced disease and those with surgical occlusion of the common bile duct reflect the essential similarity of the lesions.

The extent of obstruction to the intrahepatic bile channels and the duration of the lesions as determined by histological examination of repeated liver biopsies coincided very well with the degree and duration of impaired biliary excretion as indicated by BSP retention and serum bilirubin levels.

Significant elevations in SGOT activity occurred only in those animals with the more severe bile duct lesions among those with the hay-induced disease. Much of the elevated SGOT activity in these animals appears to be a reflection of parenchymal cell damage since degenerative

changes were observed in the hepatic cells adjacent to intensely inflamed ducts. The SGOT activity in the animals with surgically occluded bile ducts rose very rapidly, reached a high peak, and then declined rapidly during the 3-4 days immediately following surgery. Since the rise occurred so soon after surgery, it is assumed that most of the elevated SGOT in these animals was a result of surgical trauma to tissues. The SGOT activity returned to the normal presurgical levels within 10-12 days following surgery.

The nature and distribution of the hepatic lesions associated with the hay-induced photosensitivity disease described in the present investigation are strikingly similar to those reported in facial eczema of sheep (McFarlane et al., 1959; Done et al., 1960). The principle differences appear to be in severity of the lesions and sequelae. The essential lesion in both instances is an acute cholangitis which leads to massive obstruction of intrahepatic bile channels. The lesions are quite variable in degree in both diseases but tend to be more severe in facial aczema. Residual scarring along the portal tracts is usually mild in the alfalfa hay-induced disease, whereas massive biliary cirrhosis with complete obliteration of many of the intrahepatic bile channels frequently follows the acute lesion in facial eczema.

The similarity of the lesions in facial eczema and the hay-induced disease described in this report provokes some intriguing speculations concerning possible etiological similarity of the two diseases. In addition to the lesions, one other major point of similarity stands out. Levy and Smallfield (1942) noted that the incidence of facial eczema increased during warm periods in the autumn when the pasture plants were

growing luxuriantly following rains. The hay which carried the hepotoxin responsible for the present disease was grown and harvested during an unusually wet spring. Since facial eczema was shown by Thornton and Percival (1959) to be associated with a toxin produced by the saprophytic fungus, Pithomyces chartarum (syn. Sporidesmium bakeri), which develops on the pasture plants, it is tempting to speculate that a similar microbiological cause may be associated with the present disease. Limited attempts to establish such a relationship have been unsuccessful (Monlux et al., 1963).

The possibility seems remote that a toxic substance produced in the alfalfa plant itself, or in some extraneous plant in the hay, might be responsible for causing the disease in the present case. No evidence has been found which would indicate any association between the use of agricultural chemicals and toxicity of the hay.

All of the hepatogenous photosensitivity diseases share the common feature of bile retention, but the lesions and mechanisms by which bile retention occurs may be quite different.

There appears to be very little if any similarity in the nature of the hepatic lesions responsible for bile retention in the alfalfa hay-induced disease and hepatogenous photosensitivity disease other than facial eczema and possibly the bermuda grass disease which occurs in the southeastern United States. The histologic character of the lesions in the bermuda grass disease were not described but the gross lesions in the liver were described as being similar to those of facial eczema (Kidder et al., 1961).

Brown (1960) indicated that there are no bile duct lesions present

in sheep affected with Tribulosis. He suggests that the essential mechanism of bile retention may be an interference with enzyme systems which are necessary for secretion of bile into the canaliculi. A similar mechanism may be operative in Lippia poisoning (Quin, 1933b), Southdown photosensitivity (Clare, 1945), and in alveld (Ender, 1955) since no lesions of an occluding nature are described in any of these diseases. The lesions described by Mathews (1937, 1940) in Lecheguilla and Sacahuiste poisoning and those described by Clawson and Huffman (1935, 1937) in Tetradymia poisoning suggest that degenerative changes in the hepatic cells may be responsible for bile retention in those diseases.

Further work is necessary in regard to many of the hepatogenous photosensitivity diseases in order to properly characterize the hepatic lesions and to determine the specific mechanisms by which bile retention occurs.

CHAPTER VI

SUMMARY

A hepatogenous photosensitivity disease of cattle, previously proven to be caused by flood-damaged alfalfa, was further investigated and characterized. The clinical aspects of the disease, alterations in levels of various blood serum constituents and changes in liver function were compared in cattle with biliary obstruction produced by ligation of the common bile duct.

Alterations in liver function and changes in various serum components reflected the general clinical picture of the disease and followed a remarkably similar pattern in all animals regardless of the method by which the disease was induced. Significant elevations in levels of serum glutamic-pyruvic transaminase and leucine amino-peptidase did not occur in any of the animals. Elevations in serum glutamic-oxalacetic transaminase were generally of low order except in severely affected animals. Marked elevations in serum bilirubin and BSP retention occurred in direct proportion to the degree of biliary occlusion as indicated by clinical manifestation of the disease and by histologic changes in the liver.

Phylloerythrin was detectable in the blood serum only during the period of clinical photosensitivity.

The hepatic lesions associated with the hay-induced disease were

studied in biopsy and necropsy specimens from a total of 22 animals. The pathogenesis of the lesions and variable degrees of involvement are described.

The essential nature of the hepatic lesions was an acute cholangitis affecting primarily the small and intermediate interlobular bile ducts. The lesions were diffuse throughout the liver and resulted in massive intrahepatic biliary obstruction. Parenchymal lesions of significant degree were observed only in the severely affected animals. They were of a degenerative character and were confined to the zone immediately surrounding acutely inflamed bile channels. Residual lesions in the livers of recovered animals consisted of varying degrees of ductal proliferation and fibrosis of the portal tracts. These changes, however, became prominent only in cases in which ductal involvement was intense and prolonged.

The similarity of the lesions and possible etiological similarity of the alfalfa hay-induced disease and facial eczema are discussed.

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APPENDIX I

CONSUMPTION OF TOXIC HAY DURING INDUCTION PERIOD

Animal No.	Pounds Consumed per Day								Total
	Days								
	1	2	3	4	5	6	7	8	
4701	10.0	13.5	5.5	6.5	6.5	1.5	0.0	0.0	43.5
4702	5.0	13.0	11.5	4.0	5.0	5.5	1.0	0.0	45.0
4703	15.0	10.0	12.5	8.0	9.5	3.0	0.0	0.0	58.0
4704	6.0	8.5	3.0	4.5	5.0	1.0	1.0	0.0	29.0
4705	8.0	11.5	7.5	5.5	6.0	9.5	7.0	7.5	62.5
4706	10.0	8.5	15.0	13.0	12.0	3.0	7.0	6.5	75.0
Average	9.0	10.5	9.2	6.9	7.3	3.9	2.7	2.3	52.2

APPENDIX II

SERUM BILIRUBIN LEVELS IN 70 HEALTHY ADULT CATTLE

Serum Bilirubin in mg/100 ml					
Total	Conjugated	Total	Conjugated	Total	Conjugated
0.40	0.20	0.38	0.18	0.30	0.16
0.34	0.19	0.30	0.12	0.50	0.32
0.35	0.17	0.40	0.16	0.53	0.24
0.25	0.16	0.70	0.25	0.48	0.30
0.41	0.19	0.40	0.18	0.46	0.24
0.27	0.17	0.40	0.25	0.32	0.16
0.23	0.15	0.34	0.12	0.46	0.15
0.24	0.18	0.40	0.22	0.36	0.15
0.27	0.15	0.43	0.18	0.27	0.15
0.40	0.25	0.48	0.30	0.26	0.13
0.40	0.18	0.46	0.24	0.26	0.20
0.30	0.10	0.85	0.16	0.37	0.18
0.43	0.12	0.43	0.10	0.32	0.22
0.45	0.21	0.30	0.12	0.22	0.13
0.40	0.11	0.30	0.18	0.67	0.17
0.47	0.22	0.43	0.16	0.62	0.27
0.50	0.30	0.63	0.12	0.36	0.13
0.57	0.37	0.43	0.12	0.27	0.22
0.48	0.23	0.40	0.12	0.35	0.22
0.37	0.29	0.43	0.16	0.27	0.10
0.67	0.23	0.28	0.12	0.50	0.32
0.28	0.25	0.28	0.12	0.53	0.24
0.47	0.16	0.37	0.16		
0.22	0.17	0.34	0.12		

Mean Total Serum Bilirubin = 0.40 mg/100 ml, S.D. = 0.12

Mean Conjugated Serum Bilirubin = 0.19 mg/100 ml, S.D. = 0.06

APPENDIX III

HALF-TIME BSP CLEARANCE IN 62 HEALTHY ADULT CATTLE

Half-Time Clearance ($t_{\frac{1}{2}}$) in Minutes			
4.75	3.65	3.40	4.25
4.45	3.50	3.50	4.75
5.25	5.50	4.40	4.65
4.43	6.40	5.00	4.10
5.50	4.50	4.85	4.75
4.80	5.40	5.75	5.20
3.70	5.75	5.10	4.90
3.75	4.25	5.75	5.20
4.40	5.30	7.17	3.70
3.90	5.70	6.00	3.80
4.10	3.50	6.10	4.10
4.85	5.90	5.25	3.35
4.40	3.60	5.15	4.65
5.45	4.45	5.70	3.75
4.50	4.25	4.10	
4.60	4.10	4.50	

Mean Half-Time Clearance = 4.70 Minutes, S.D. = 0.83

APPENDIX IV

SERUM ENZYME ACTIVITY IN 64 HEALTHY ADULT CATTLE

SGOT Sigma-Frankel Units				SGPT Sigma-Frankel Units				LAP G-R Units			
53	78	100	78	11	29	23	11	18	29	18	26
41	71	78	74	9	17	17	8	20	31	20	26
50	71	100	78	17	26	17	20	18	26	20	26
58	71	86	86	14	23	17	15	25	31	30	26
46	24	94	86	23	26	15	25	20	22	28	30
90	68	68	86	20	23	13	23	22	22	22	30
68	70	104	86	24	26	9	11	16	18	26	30
58	53	78	78	16	20	11	18	20	18	30	30
48	53	70	104	20	20	14	18	16	33	30	30
95	58	64	100	15	23	14	15	25	22	22	26
64	78	58	104	17	23	15	13	25	26	33	30
58	41	64	74	23	20	16	11	23	29	26	26
46	53	70	70	23	23	11	9	20	38	35	26
90	58	104	90	23	23	7	8	22	26	30	26
70	50	94	86	15	17	13	15	18	18	35	26
74	64	74	70	26	15	14	15	26	18	26	26
Mean S-F Units = 71 S.D. = 20				Mean S-F Units = 17 S.D. = 5				Mean G-R Units = 25 S.D. = 5			