INVESTIGATIONS IN LABORATORY ANIMALS OF A BOVINE PHOTOSENSITIZATION SYNDROME ASSOCIATED WITH THE CONSUMPTION OF MOLDY, ALFALFA HAY

by

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INTRODUCTION

In January of 1971, an outbreak of photosensitization occurred on a farm in Home, Kansas. It affected approximately two-thirds of a herd of 160 Shorthorn cattle. The animals were being fed silage and visibly moldy alfalfa hay with rank soil bank growth in it. Subsequent analysis of the hay for aflatoxins revealed the equivalent of 1,704 ppb aflatoxin B_1. A species of the fungus Alternaria was cultured from the hay. Similar outbreaks of photosensitization associated with alfalfa hay or moldy alfalfa hay have occurred sporadically in this area of the United States.[24, 25, 26, 34, 36]

Photosensitization may be defined as excessive sunburning upon exposure to sunlight. A photosensitizer or "photodynamic agent" enters the skin by way of the peripheral circulation. This photosensitizer becomes activated by light and transfers its energy of activation to other molecules (proteins and enzymes) which then become oxidized and damaged in the presence of oxygen.[3, 12, 13]

The early signs of photosensitization are pruritus and erythema with the affected animal shaking its head and seeking shade. The unpigmented areas of the skin, especially lightly haired areas, are most affected. Signs are first noticed in the ears and around the mouth, if unpigmented. Erythema may progress to edema and cracking of skin with seeping of edematous fluid. Eventually the affected areas may slough; secondary infections often set in. Severity

of signs is dependent on the intensity of the sunlight and the length of exposure. Affected animals usually recover if removed from the source of exposure and kept indoors.

Depending on the source of the photosensitizer in the skin, photosensitization can be divided into three main types: Congenital photosensitization, primary photosensitization, and hepatogenous photosensitization. Congenital photosensitization is an "inborn defect" whereby photosensitizing porphyrins increase in the body because of excessive production. Primary photosensitization is due to the ingestion of a pigment, not normally ingested, which is absorbed into the bloodstream. Four sources of the pigment are possible: (1) Drugs, such as phenothiazine and sulfonamides, or their metabolites, which possess photosensitizing properties; (2) Fluorescent dyes, such as eosin and methylene blue, which are injected into the body (this type of photosensitization occurs only experimentally); (3) Plant pigments, such as hypericin found in the plant St. John's Wort; and (4) Fungal pigments. Hepatogenous photosensitization, often accompanied by icterus, occurs when sufficient hepatic damage is present to interfere with bile flow. Phylloerythrin, a breakdown product from the bacterial digestion of chlorophyll then accumulates in the body. Normally, this pigment is detoxified by the liver and is not present in the peripheral circulation in sufficient concentration to cause photosensitization. When it does increase

sufficiently, photosensitization results upon exposure to sunlight.

In the described outbreak, congenital photosensitization was eliminated because of the age of the animals, the large number of animals involved, and the suddenness of the onset. Since there was no evidence in the history of a possible drug-induced photosensitization, the cause was either primary, due to a plant or mold pigment, or hepatogenous, due to initial liver damage and accumulation of a photosensitizing agent.

All harvested grains, forage, or other feedstuffs are suitable media for the growth of a wide variety of molds, provided temperature and moisture conditions allow growth. Thus, feedstuffs may frequently have detectable and very substantial mold populations.[10] The discovery that aflatoxins are produced by certain strains of the mold Aspergillus flavus[40] focused attention on the possibility that other seemingly inocuous molds might also produce toxins.

Although there is no reference in the literature incriminating aflatoxins of themselves as causing photosensitization, aflatoxins do produce a primary liver damage that could lead to hepatogenous photosensitization. The typical liver lesions produced by aflatoxins include hepatic necrosis with subsequent bile duct proliferation. Severe fibrosis is usual in calves and pigs.[65]

The implication that many plants cause photosensitization when moldy or under conditions when mold growth is likely, suggests that many outbreaks of photosensitization may be due to molds or their toxins. The role that mycotoxins play in this process is, as yet, not understood.

In an effort to learn more about this condition, a study was made to determine if signs of photosensitization could be produced in laboratory animals. The alfalfa hay associated with the cited photosensitization in cattle was fed to mice, guinea pigs, and rabbits. Liver damage was induced in some of the experimental animals to evaluate the potential role of this variable in the occurrence of photosensitization.

LITERATURE REVIEW

The most studied mold-induced photosensitization is facial eczema, a disease of grazing sheep which occurs in New Zealand in the autumn months. For years, it was thought to be caused by rye grass, growing under abnormal climatic conditions. The liver damage associated with this disease is a severe obliterative cholangitis with subsequent bile duct hyperplasia, fibrosis, and atrophy of the inner parenchyma.[15] Facial eczema was classified as a hepatogenous type of photosensitization when it was determined that phylloerythrin was present in the blood of sheep with facial eczema in sufficient amounts to produce the clinical signs.[11]

Liver lesions similar to those in sheep with facial eczema have been produced in guinea pigs fed on grass collected and dried during an outbreak of the disease. After 28 to 50 days, the guinea pigs showed progressive cholangio-obstructive changes associated with massive ductule proliferation. This established the guinea pig as a test animal for facial eczema.[19] Photosensitization was produced (using artificial light) in a guinea pig fed for 7 days on toxic grass extract, followed by green grass ad lib for 2 additional days. Photosensitization was also produced in a guinea pig grazed outdoors on toxic grass for 8 weeks. Further experiments established that rats and mice were not satisfactory test animals for work on facial eczema.[39]

The rabbit was found to be more susceptible than the guinea pig to facial eczema. The liver lesions were similar to, but more severe than, those seen in guinea pigs and lambs. Edema of the extra-hepatic ducts was more frequently seen. However, no attempt was made to produce photosensitization in the rabbit.[14, 16]

The fungus, Pithomyces chartarum produced liver lesions in sheep and guinea pigs similar to those caused by feeding toxic grass collected during an outbreak of facial eczema.[33, 38, 45] Signs similar to those seen in the guinea pig and lamb were produced by feeding rabbits the spores of Sporidesmium bakeri (P. chartarum) and ether extracts of toxic grass or cultures of S. bakeri.[14] The toxin, sporidesmin, was isolated from the same fungus.[42, 43] The liver damage in guinea pigs produced by sporidesmin was identical to that produced by wet cultures or spores of S. bakeri or by toxic pasture.[43]

A photosensitization syndrome in the United States has occurred in the states of Florida, Georgia, and Texas during the months of December, January, and February, usually 3 to 8 weeks following a frost. In these cases, Bermuda grass or clover became matted and molded when damaged by frost or by drought, followed by rain.[23, 30, 41] The responsible mold was identified as Periconia minutissima Cla. and the syndrome was believed to be essentially a mold toxicity.[30] The mold produced an icterogenic substance in

the forage which damaged the liver and prevented the elimination of phylloerythrin and bile.[23, 30] The same fungus responsible for facial eczema, P. chartarum, was recovered from grasses in Texas coastal plains pastures where photosensitization was a problem to livestock production and where signs had appeared after dry spells followed by rain and rising temperatures. The relationship between this fungus and the frosted Bermuda grass photosensitization has yet to be established.[44] P. chartarum has also been isolated from seed and fruit in Oregon.[32]

It was observed that the fungus Penicillium viridicatum produced hepatic lesions in mice which resembled those in facial eczema lambs. A marked pericholangetic inflammatory exudate was accompanied by a necrotizing cholangitis and choleocystitis. Following the acute lesions, granulation tissue formed with partial stenosis or obliteration of biliary ducts.[4, 8] Photosensitization was induced in mice by feeding rice cultures of P. viridicatum. The microscopic changes in the ears and face resembled those in sheep with facial eczema, cattle with the Bermuda grass toxicosis, and cattle with the hepatogenous photosensitization associated with the feeding of alfalfa hay. Because of the microscopic liver lesions, the syndrome was classified as a hepatogenous photosensitization. Since the mice were fed a chlorophyll-free diet, it is unlikely that phylloerythrin was the photodynamic agent. It was speculated that circulating

biliary porphyrins or pigments produced by the fungus were the photodynamic chemicals.[5]

Although alfalfa, clover, and other legumes are ordinarily regarded as harmless, there are many statements in the literature that they have occasionally been suspect of producing photosensitization.[3, 9, 12, 17, 27, 29, 31, 35, 46, 47]

Actual case descriptions of photosensitizations involving clovers are numerous. In New South Wales, newly shorn sheep placed on trefoil pasture developed signs of photosensitization in one week. Black sheep and sheep on trefoil-free pasture were not affected.[37] A case in cattle was diagnosed as photosensitization by subterranean clover.[20] Horses became photosensitive to alsike clover (Trifolium hybridum) in full bloom. Sheep and heifers on surrounding pastures were not affected.[21] In one of the few experiments performed, trefoil dermatitis was produced in guinea pigs by feeding them Medicago denticulata. The cause was believed to be due to the ingestion of fungi with the clover.[17] A case was described in Australian sheep on M. denticulata or M. apiculata. M. denticulata and M. confinis were collected from the banks of a small stream previously flooded and fed to 2 guinea pigs. However, they remained normal when exposed to sunlight.[6]

Clovers and other legumes may contain substances which cause liver injury or some depression of liver function, thereby preventing the normal removal of the photosensitizing

phylloerythrin from the peripheral circulation.[35] Another opinion is that trefoil dermatitis may belong to the group of primary photosensitizers.[12, 13]

Alfalfa, or lucerne, has been involved in several cases of photosensitization. An outbreak in pigs on M. denticulata and lucerne (M. sativa) has been described.[7] Many cases of "Geeldikkop" in South African sheep developed in sheep grazing grasses and alfalfa in the absence of the weed, Tribulus terrestris, commonly believed to be responsible.[30, 35] Cases occurred in Hereford cattle in Kansas. The diet consisted of brome-grass pasture and supplementary alfalfa.[36] A hepatogenous photosensitization was recorded in a herd of Herefords and a herd of Holsteins which were fed a first cutting of alfalfa hay for 6 weeks.[2]

An outbreak was described which involved Guernseys with access to native grass pasture, weeds, and a bordering wooded area. Heavy frost during the 10 days preceding the outbreak had damaged the vegetation. Five to 7 days prior to the disease onset, the diet was supplemented with mixed alfalfa and oats. In this outbreak, considerable variation was noticed in toxicity from different parts of a field, as well as from different fields. The toxicity of the hay remained at or near its original level for 3 years. Although species of several genera of fungi were identified, none were known tc possess hepatoxic properties. Spores indistinguishable from those of P. chartarum were observed in

washings from the plants, but attempts to culture the organism were unsuccessful. The unusual climatic conditions were favorable for excessive growth of fungi on vegetation. Similar conditions were associated with excessive growth of P. chartarum.[34]

The case described above is from the outbreak of photosensitization which occurred in the fall and winter of 1957-1958 in the eastern two-thirds of Oklahoma and adjacent areas of bordering states. In all instances, the disease was associated with the feeding of first-cutting alfalfa hay grown on lowlands along streams. The alfalfa had been damaged by heavy rains and flooding prior to harvest during the unusually wet spring of 1957. The flood-damaged hay was fed to cattle. The clinical aspects of the hay-induced disease were compared with the photosensitization caused by surgically-induced biliary obstruction. The majority of animals fed the hay and all those with induced obstruction developed photosensitivity which was regularly accompanied by icterus. Lesions of photosensitization were variable in severity among individual animals, but were essentially identical in the hay-induced and surgically-induced disease.[25] The biliary tract was the major site of action for the toxic agent involved in the syndrome. Occlusion of the lumens of most small and intermediate bile ducts, caused by epithelial hypertrophy and hyperplasia and by the inflammatory changes which occurred, offered presumptive

evidence that the functional disturbance was mechanical obstruction to bile flow.[26] Phylloerythrin was probably the photodynamic agent since it was present in serum only during the period of clinical photosensitivity.[24] Neither the signs nor the lesions developed to the extreme degree described with the more severe cases of facial eczema. However, the similarity of the milder lesions of facial eczema and those of the alfalfa hay disease warranted serious consideration of a similar, if not identical, etiological relationship between the two diseases. The nature of the toxin responsible for the liver lesions in the alfalfa hay disease remains unknown. Since the lesions were confined almost exclusively to the biliary tract, the toxin was apparently concentrated and eliminated via the biliary secretion mechanisms without being adequately neutralized by detoxification processes of the liver.[26] Feeding hay from the same outbreak to guinea pigs, rabbits, mice, rats, or hamsters did not produce obstructive biliary lesions or photosensitivity.[34]

MATERIALS AND METHODS

Experimental Hay.---The experimental hay used in this study was the same hay that caused the outbreak of photosensitization described in the introduction. It was obtained from the owner of the herd on January 7, 1972, approximately one year after the outbreak. During this year it had been left outdoors entirely exposed to the weather. It was dry, weedy, and visibly moldy, especially on the outer surface of the bales.

The hay was cultured for fungi in the following manner: Several clippings from the most moldy areas of the hay were placed on Sabouraud's dextrose[a] agar plates and incubated at 25 C for 4 days. Each distinct colony was then subinoculated on Mycobiotic[b] agar slants and incubated at 25 C for 1 week. Colonies were checked for purity by preparing teased mounts of the culture in lactophenol cotton-blue. Slide cultures of Mycobiotic agar were prepared for each isolate and incubated at 25 C for 1 week. Each isolate was identified according to genus by microscopic characteristics of the mycelia and spores.

Control Hay.---The hay which served as the control hay in this experiment was obtained from the Animal Research

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[a] Bacto-Sabouraud's Dextrose Agar, Difco Laboratories, Detroit, Mich.

[b] Bacto-Mycobiotic Agar, Difco Laboratories, Detroit, Mich.

Facilities, Kansas State University. It was a good quality green alfalfa hay which was being fed to the sheep and cattle.

Aflatoxin Assay.---Aflatoxin assay was done on the experimental hay, the control hay, and the experimental animals' litter. The 19 x 300 mm. silica gel column[c] used in the assay was packed as follows: Five Gm. of granular anhydrous sodium sulfate were added to the column. Ten Gm. of silica gel[d] were dispersed in 150 ml. of chloroform by stirring. This suspension was then added to the column. Settling was aided by drawing off some of the chloroform, but leaving 5-8 cm. of chloroform above the silica gel. Fifteen Gm. of sodium sulfate were slowly added above the silica gel partition and the chloroform was drawn off to the top of this layer. The aflatoxin assay proceeded as follows: Fifty Gm. of hay or litter were placed in a blender; 250 ml. of 70% acetone were used to blend the hay and transfer it to a 250 ml. frosted-neck Erlenmeyer flask. The flask was stoppered and shaken on a mechanical shaker[e] for 30 minutes. The contents were filtered through filter paper[f] in a Buchner funnel. To 150 ml. of the filtrate, 20 ml. of 20% neutral

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[c] Model 274-019, Fisher and Porter Co., Warminster, Pa.

[d] Silica gel, 0.05-0.20 mm., E. Merck Ag, Inc., Darmstadt, Germany.

[e] Burrell "Wrist Action", Burrell Corporation, Pittsburgh, Pa.

[f] Whatman No. 42, 7 cm., W & R Balston, Ltd., England.

lead acetate and 60 ml. of deionized water were added and the mixture was stirred. This was boiled in a water bath until it had evaporated to 150 ml. volume. The contents were then transferred to centrifuge tubes and centrifuged at 1,300 r.p.m. for 30 minutes. The supernatant was decanted into a 250 ml. separatory funnel. The precipitate was resuspended in a total of 60 ml. of 20% aqueous acetone solution and recentrifuged for 10 minutes. This supernatant was added to that already in the separatory funnel. The combined supernatants were shaken vigorously with 50 ml. of chloroform for one minute. The phases were allowed to separate, and the lower phase was collected in a 250 ml. beaker. This step was repeated once. The fluid in the beaker was boiled in the steam bath until the volume had evaporated to 2-3 ml. This fluid was pipetted into the prepared silica gel column and drawn into the column. Then, 150 ml. of hexanes, followed by 150 ml. of anhydrous diethyl ether, were passed through the column and discarded. Finally, 150 ml. of 3% methanol-chloroform solution were eluted through the column and collected in a 250 ml. beaker. This solution was reduced to dryness in the water bath. One-half ml. of chloroform was added to the dry sample extract. Using a 10 ul. automatic pipette,[g] the samples were spotted at 2 cm. intervals along a line 2 cm. from

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[g] Oxford Sampler, Oxford Laboratories, San Mateo, Cal.

the bottom edge of a pre-coated silica gel sheet on aluminum[h] which had been baked in a 125 C oven for 1 hour to remove moisture. For each extract, the following were spotted: 5 ul. of the extract, 10 ul. of the extract, 5 ul. of the extract plus 5 ul. of the aflatoxin standard, and 10 ul. of the aflatoxin standard. The quantitative aflatoxin standard was prepared from pure crystalline aflatoxin B_1 and G_1[i] which was dissolved in chloroform so that 1 ml. of the solution contained 0.5 ug. each of aflatoxin B_1 and G_1. The standard was placed in a tightly sealed vial wrapped in aluminum foil and stored at 0 C until used. The spotted plate was allowed to air dry for 10 minutes and was then developed in a chromogram chamber[j] with a trough containing 50 ml. of acetone:chloroform (1:9) solution until the solvent front had traveled 15 cm. from the point of sample application. After developing, the sheet was allowed to air dry and was observed for fluorescing spots under a long range ultraviolet light.[k] Aflatoxin quantitation was made visually by comparing the intensity of fluorescence of the unknown to that of the standard, using the formula: ug. aflatoxin/l. (or ppb aflatoxin) = (S)(Y)(V)/(X)(W); where

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[h] E. Merck Ag, Inc., Darmstadt, Germany.

[i] Makor Chemicals, Ltd., Israel.

[j] Eastman Chromogram Chamber Plate Set, Distillation Products, Inc., Rochester, N. Y.

[k] Mineralight, Model UV-25, Ultraviolet Products, Inc., San Gabriel, Cal.

S = ul. of aflatoxin standard equal to the unknown; Y = concentration of the aflatoxin in the standard solution; V = the volume in ul. to which the sample extract was finally diluted; X = ul. of the sample extract spotted giving fluorescence equal to S the aflatoxin standard; and, W = the grams of sample originally used times 0.6.

Experimental Animals.---The rabbits[l] used in these experiments were New Zealand White females, weighing from 200 to 700 Gm. upon arrival. They were 4-5 weeks of age at the beginning of the experiment. The guinea pigs[m] were white American Shorthair females, weighing approximately 200 Gm. at the beginning of the experiment. Both the rabbits and the guinea pigs were weighed daily. They were marked for identification by clipping hair from various areas of their bodies. The strain of mice[n] was Outbred:NAT NLW (sw). They were approximately 5 weeks of age when obtained. They were housed with wood shavings for litter. All experimental animals were housed out of direct sunlight.

Diet Formulation.---To make the hay edible for the animals, the experimental hay was ground in a hammer mill. The ground hay was formulated into a diet consisting of pancake flour[o]

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[l] Alve Louk Rabbitries, Manhattan, Ks.

[m] Pel-Freez Bio-Animals, Inc., Rogers, Ark.

[n] Animal Diagnostic Laboratory, Kansas State University, Manhattan, Ks.

[o] Hungry Jack Buttermilk Pancake and Waffle Mix, Pillsbury Co., Minneapolis, Minn.

and ground hay mixed as 25, 50, 75, or 90% ground hay. Molasses[p] and water in equal amounts were added to moisten the hay and to improve palatability. The control hay was ground and formulated into a diet in an identical manner.

Experimental Design.---The experiments were performed as short-term (1 week) feeding trials and long-term (at least 4 weeks) feeding trials in rabbits, guinea pigs, and mice. Short- and long-term trials in mice with induced liver damage were also done. Liver damage was produced by 1 injection of 0.25 ml. carbon tetrachloride (CCl_4) subcutaneously at the end of the feeding trial. The mice were fed the 25, 50, 75, or 90% hay diets as indicated in Table 1. The rabbits and guinea pigs were fed the 75% hay diets exclusively (Table 2) because with this diet they received the optimal combination of a high percentage of alfalfa hay with enough other nutrients to make the diet palatable and to allow them to increase their body weights.

At the end of each feeding period, the animals were exposed to direct sunlight for 2 hours, usually from 11:00 a.m. to 1:00 p.m. In the case of animals with induced liver damage, sun exposure was performed two days after CCl_4 injection. Animals had free access to food and water during the exposures. The sun exposures were carried out in

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[p] Brer Rabbit New Orleans Molasses, Green Label, RJR Foods, Inc., New York, N. Y.

early spring and summer. Animals were exposed for one day only, except for the long-term rabbits and the long-term guinea pigs, which were exposed on several days (Tables 1 and 2). All animals were observed for 24 hours for signs of photosensitization and then euthanatized. Mice were exsanguinated under ether anesthesia by cutting into the infraorbital sinus of the eye with a scapel. Guinea pigs and rabbits were euthanatized with pentobarbital sodium[q] injected intrathoracically or intracardially after blood samples had been collected via intracardial puncture. Plasma was collected from the mice in the following manner: Heparinized[r] syringes and needles were used to collect blood from the infraorbital sinus. The blood was placed into polyethylene tubes[s] and centrifuged for 10 minutes. Plasma was drawn off with Pasteur pipettes, placed in polyethylene tubes, capped, and frozen at 0 C until used.

A portion of the right diaphragmatic lobe of the liver was obtained immediately after death from all animals except the long-term mice. The tissue was fixed in buffered neutral 10% formalin for at least 48 hours before processing. Histological sections were prepared from paraffin tissue blocks, sectioned at 6 u., and stained with hematoxylin and eosin (H & E).

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[q] Barb-Euthol, Haver Lockhart Laboratories, Shawnee, Ks.

[r] Lipo-Heprin, 1,000 units/ml. Riker Laboratories, Inc., North Ridge, Cal.

[s] Microtest-tubes, Beckman Instruments Inc., Fullerton, Cal.

Serum Glutamic-Pyruvic Transaminase (SGPT) Testing.--- SGPT testing was done on all the guinea pigs, rabbits, and short-term mice. In the short- and long-term mice injected with CCl_4, sufficient SGPT levels were determined to verify liver damage. In a preliminary experiment in long-term mice given 2 injections of CCl_4 and then sun-exposed, SGPT values were found to be increased (Appendix V). SGPT values were also determined for 6 normal, non-experimental mice to estimate normal values.

SGPT testing was done using the method of Wroblewski-LaDue[49] as modified by Henry et al.[28] The frozen plasma samples were thawed at room temperature for 30 minutes. A SGPT reagent tablet[t] was dissolved in 3 ml. of distilled water and heated at 37 C for 20 minutes. Fifty ul. of plasma were pipetted into the solution with a micropipette. After 10 minutes, the first absorbance reading was taken at 340 nm. on the spectrophotometer.[u] Another reading was taken exactly 15 minutes later. The difference between the two readings was multiplied by the factor of 1,333 to quantify the SGPT value in spectrophotometric units. This value was multiplied by 0.48 to secure the value in International Units (I.U.).

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[t] Eskalab Reagent Tablets, Smith Kline Instruments, Inc., Palo Alto, Cal.

[u] Eskalab Spectrophotometer "Alpha", Smith Kline Instruments, Inc., Palo Alto, Cal.

TABLE 1---Feeding Trials in Mice

Type of feeding trial	Hay formulation	Number of days on hay	Number of animals	Sex	Date of sun exposure	Age at exposure (weeks)
Short-term	25% Experimental	7	5	F	1 June	6
	25% Control	7	2*	F	1 June	6
	50% Experimental	7	5**	M	1 June	6
	50% Control	7	2	F	1 June	6
	75% Experimental	7	5	F	1 June	6
	75% Control	7	2	F	1 June	6
	90% Experimental	7	4	F	1 June	6
	90% Control	7	2	F	1 June	6
Short-term plus CCl_4 liver damage	25% Experimental	7	6*	F	1 June	6
	25% Control	7	2*	F	1 June	6
	50% Experimental	7	6**	M	1 June	6
	50% Control	7	2	M	1 June	6
	75% Experimental	7	6*	M	1 June	6
	75% Control	7	2*	M	1 June	6
	90% Experimental	7	6*	F	1 June	6
	90% Control	7	2	F	1 June	6

* One or ** two animals in this group died prior to termination of the experiment.

TABLE 1---Feeding Trials in Mice (continued)

Type of feeding trial	Hay formulation	Number of days on hay	Number of animals	Sex	Date of sun exposure	Age at exposure (weeks)
Long-term	25% Experimental	33	8	F	16 April	10-13
	25% Control	33	2	F	16 April	10
	50% Experimental	31	8	F	14 April	13
	50% Control	31	2	F	14 April	10
	75% Experimental	28	8	F	5 April	12
	75% Control	28	2	F	5 April	12
	90% Experimental	30	8	F	22 April	11
	90% Control	30	2	F	22 April	14
Long-term plus CCl_4 liver damage	25% Experimental	43	6	F	30 June	11
	25% Control	43	2	F	30 June	11
	50% Experimental	43	6	M	30 June	11
	50% Control	43	2	M	30 June	11
	75% Experimental	43	6	F	30 June	11
	75% Control	43	2	F	30 June	11
	90% Experimental	43	6	M	30 June	11
	90% Control	43	2	M	30 June	11

TABLE 2---Feeding Trials in Guinea Pigs and Rabbits

Species	Type of feeding trial	Hay formulation	Number of days on hay	Number of animals	Sex	Date(s) of sun exposure	Age at exposure (weeks)
Guinea pig	Short-term	75% Experimental	8	4	F	22 May	4½-5½
		75% Control	8	2	F	22 May	4½-5½
	Long-term	75% Experimental	28	4*	F	11,12,15, 17,18 June	7½-8½
		75% Control	28	2	F	11,12,15, 17,18 June	7½-8½
Rabbit	Short-term	75% Experimental	7	4	F	1 June	5½-6½
		75% Control	7	2	F	1 June	5½-6½
	Long-term	75% Experimental	34	4*	F	22-28 June	9½-10½
		75% Control	34	2	F	22-28 June	9½-10½

* One animal in this group died prior to termination of the experiment.

RESULTS

Mycological Culture of the Experimental Hay.---The fungi isolated from the experimental hay were: Scopulariopsis sp.; Cladosporium sp.; Penicillium sp., and 2 different species of Alternaria.

Aflatoxin Assays.---Aflatoxin assay of the experimental hay revealed 750 ppb aflatoxin B_1 and 750 ppb aflatoxin G_1 for a total of 1,123 ppb B_1 equivalent. The control hay had 16.67 ppb aflatoxin B_1 and 16.67 ppb aflatoxin G_1 for a total of 25.01 ppb B_1 equivalent. The litter used in the cages of the experimental animals contained no detectable aflatoxins. The detection limit of the assay method was 0.5 ppb.

Experimental Animals.---After injection of the CCl_4, some of the mice developed large swellings on their backs. When opened, these swellings were air pockets with thickened skin and serum exudate.

Mice accepted the hay diets well, although those on high percentages of hay were at first reluctant to eat. Acceptance of the hay diets was fair among the rabbits and poor among the guinea pigs. All of the rabbits maintained or increased their body weights (Figures 1-3, Appendices I-II). Most of the guinea pigs lost a small amount of their original body weight (Figures 4-6, Appendices III-IV), but this occurred in both experimental and control groups.

During sun-exposure on hot days, mice occasionally became frantic and tried to escape from their cages. This occurred in control and experimental mice. Guinea pigs and rabbits would occasionally shake their heads, but made no attempt to escape or seek shade. Both the experimental and control guinea pigs and rabbits developed red ears after sun-exposure, but no aural lesions resulted. Signs of photosensitization did not occur in any of the experimental animals.

Liver Pathology.---Liver lesions were only seen in the mice with CCl_4-induced liver damage. These mice had pale livers with marked accentuation of the normal lobular pattern. The microscopic changes in the CCl_4-damaged livers consisted of moderate to severe centrilobular necrosis of the parenchymal cells.

No significant histological lesions were noted in any of the other experimental or control animals, and there was no pathological difference between the animals on experimental or control hay.

SGPT Values.---The SGPT values for 6 control mice on a normal ration were: 7.68; 9.60; 13.44; 14.72; 17.92, and 24.96 I.U. The mean and standard deviation of these values was 14.72 ± 5.66.

The SGPT values and means for the short-term mice, guinea pigs, and rabbits are listed in Tables 3, 4, and 5 respectively. SGPT values and means for the mice injected once with CCl_4 are given in Table 6.

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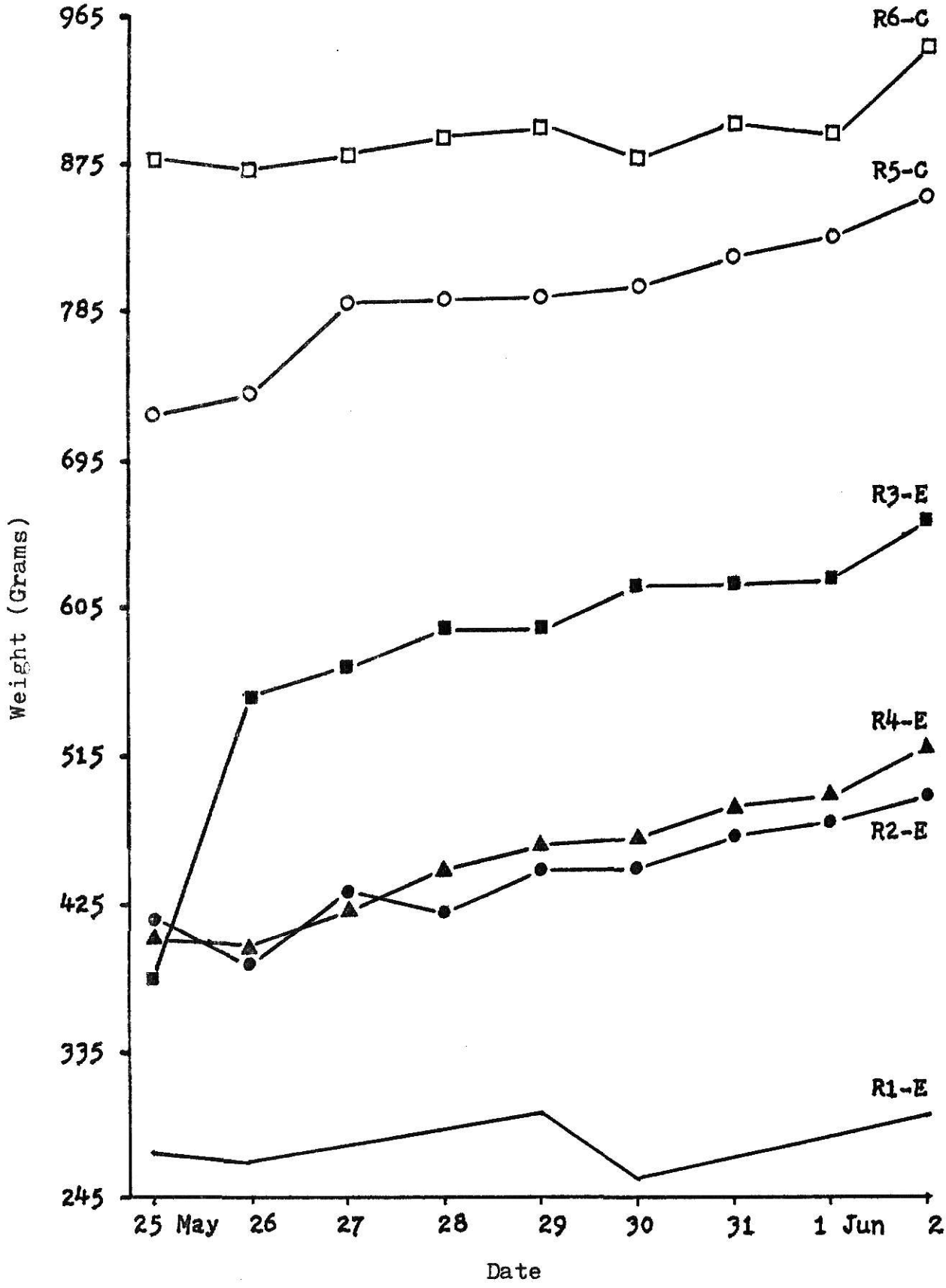

Figure 1---Daily Weights of Short-Term Rabbits.

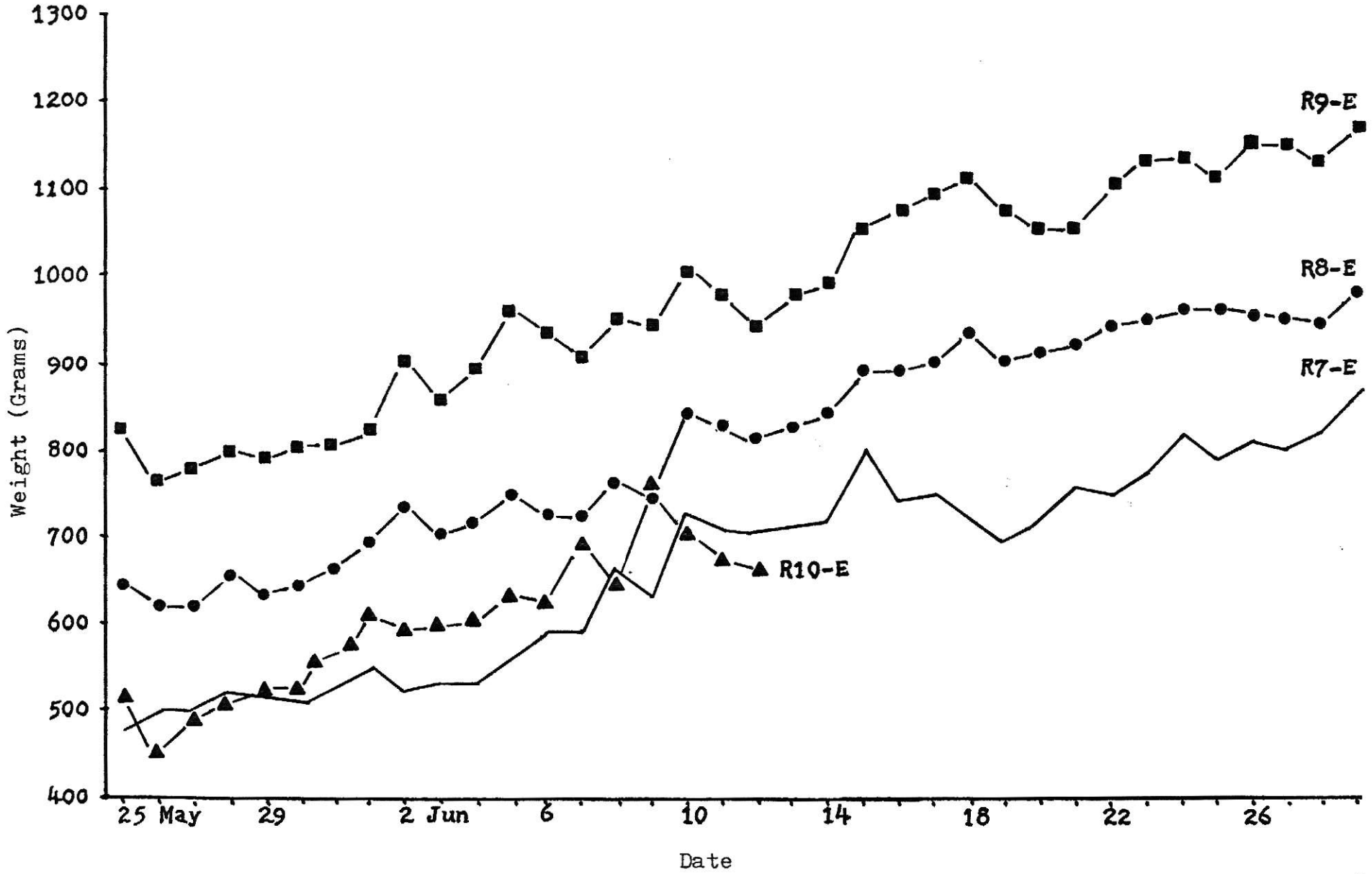

Figure 2---Daily Weights of Long-Term Rabbits on Experimental Hay.

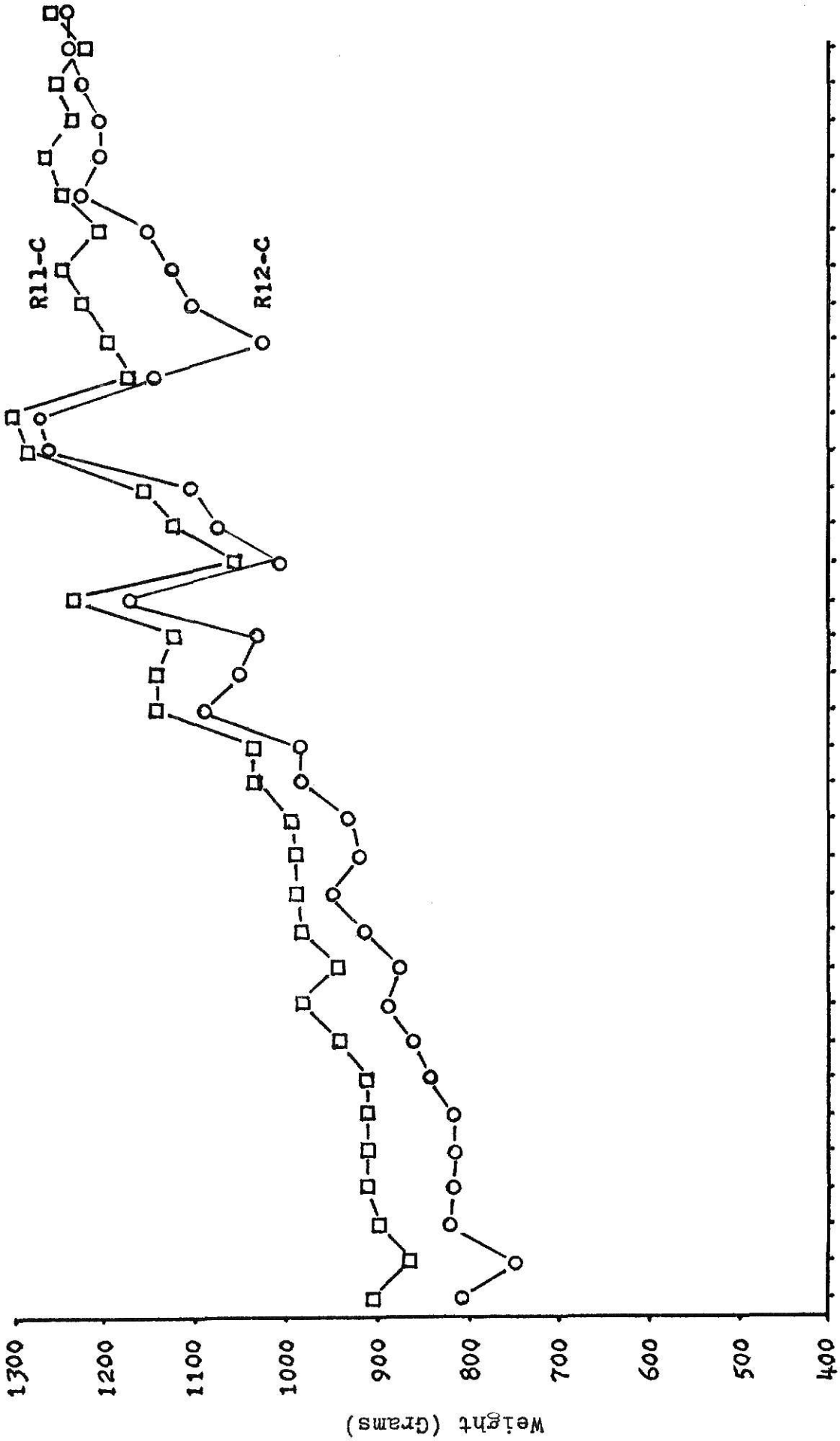

Figure 3---Daily Weights of Long-Term Rabbits on Control Hay.

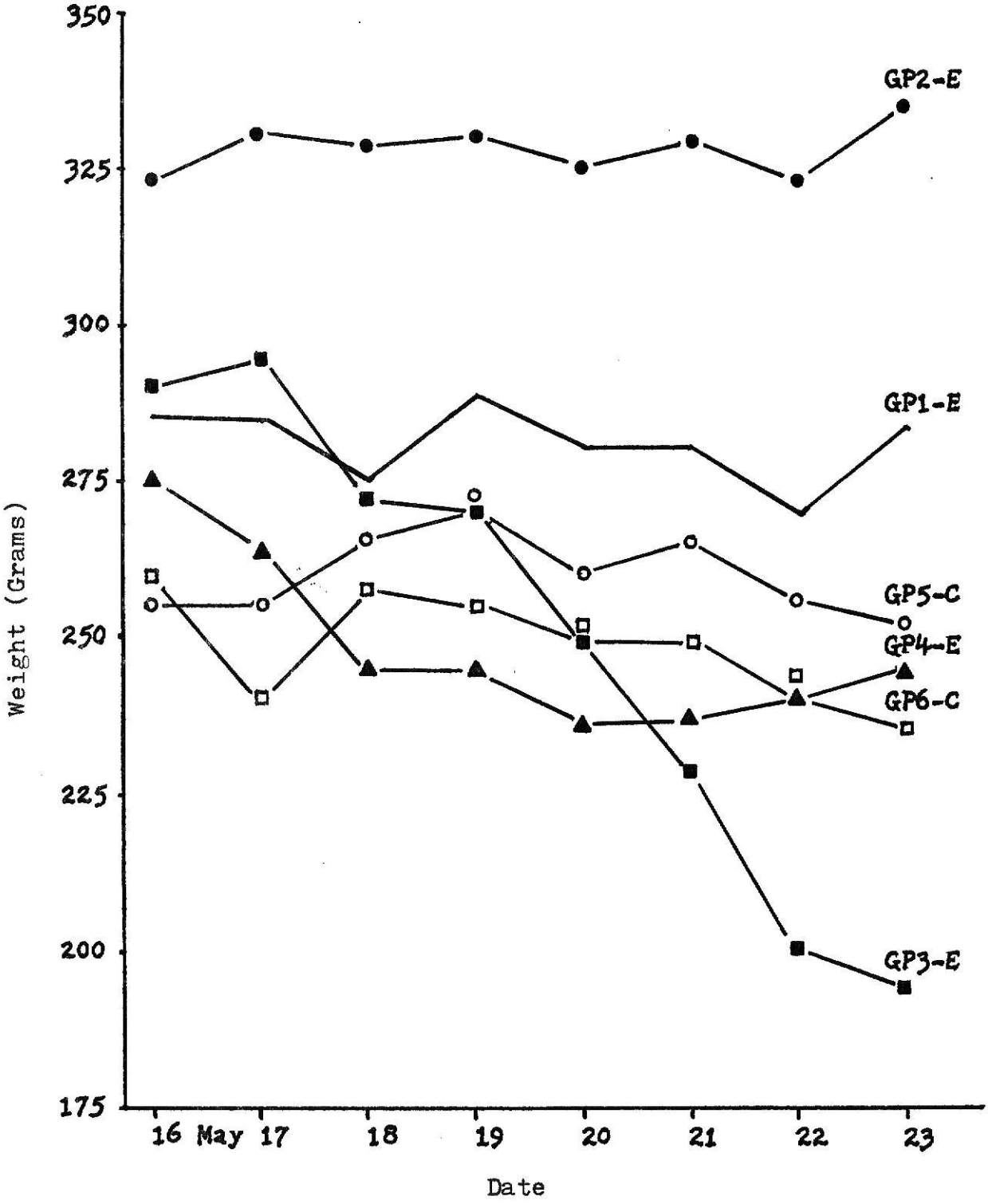

Figure 4---Daily Weights of Short-Term Guinea Pigs.

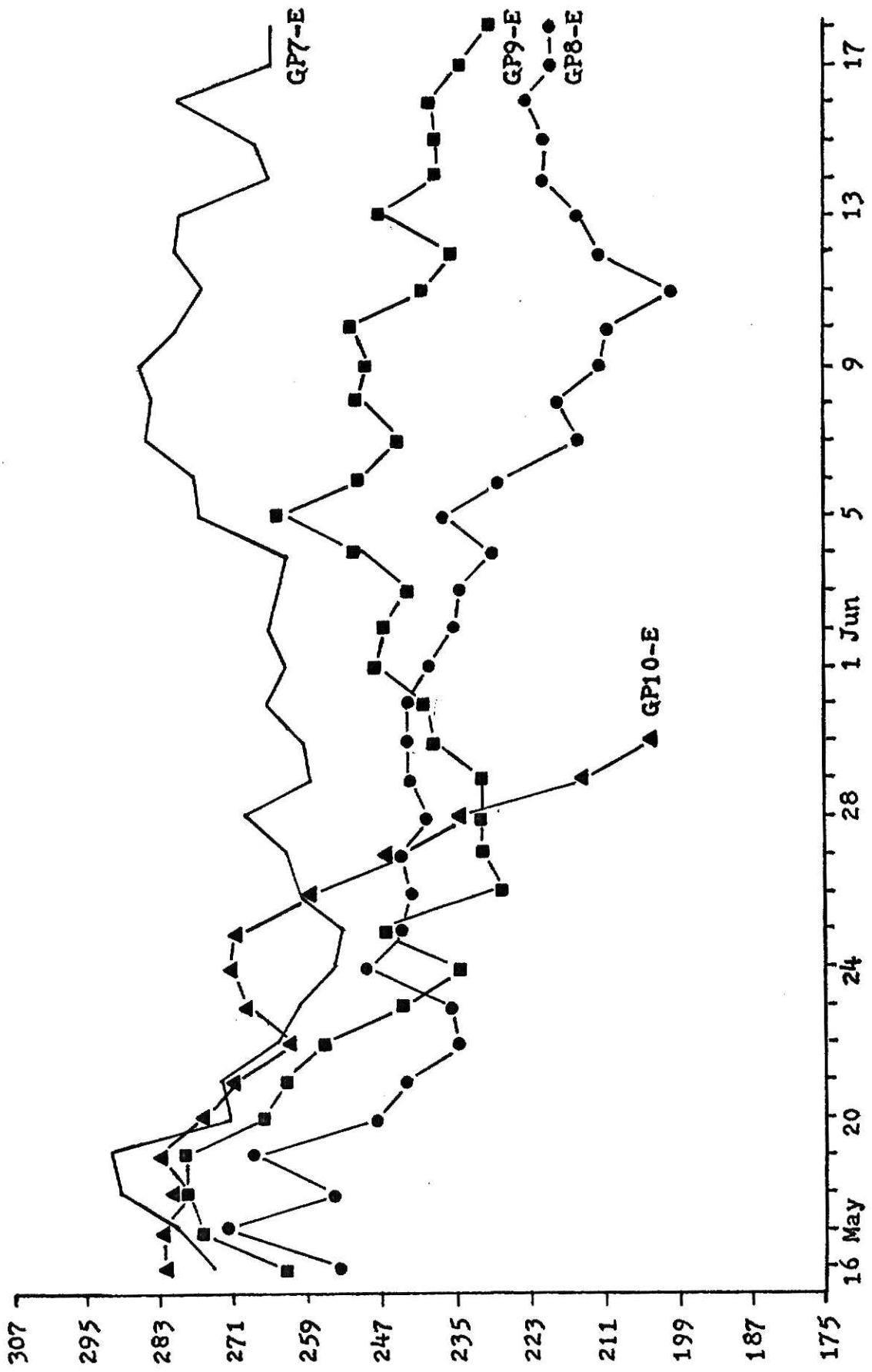

Figure 5---Daily Weights of Long-Term Guinea Pigs on Experimental Hay.

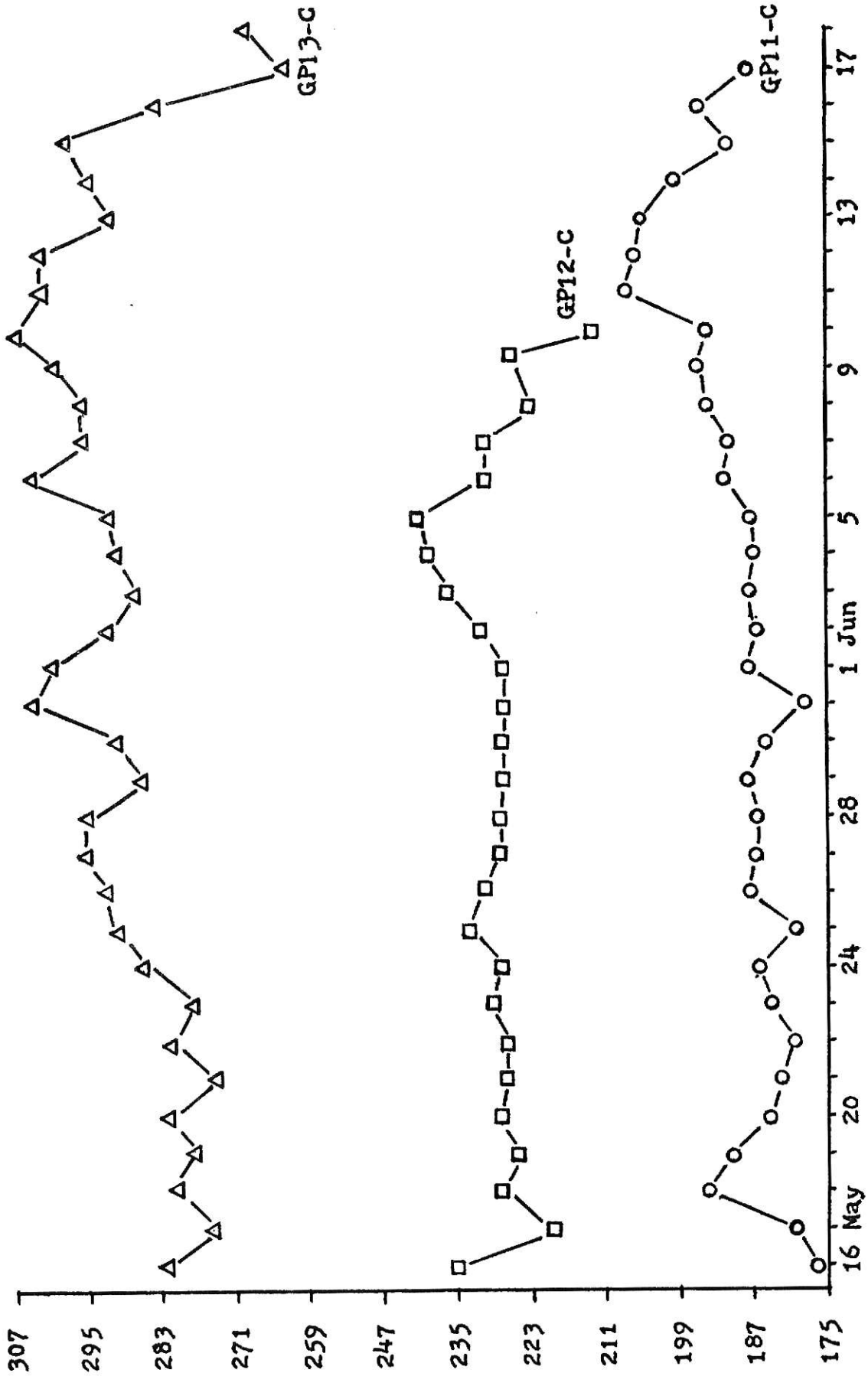

Figure 6---Daily Weights of Long-Term Guinea Pigs on Control Hay.

TABLE 3---SGPT Values for Short-Term Mice

Hay formulation	SGPT (I.U.)	Mean	Standard deviation
25% Experimental	4.48 4.48 5.12 7.68 8.32	6.01	1.65
25% Control	7.68	7.68	---
50% Experimental	15.36 15.36 17.92	16.21	1.21
50% Control	18.56 26.23	22.40	3.84
75% Experimental	4.48 5.12 5.76 5.76 6.40	5.50	0.65
75% Control	5.76 23.03	14.40	8.64
90% Experimental	6.40 8.32 8.32 10.88	8.48	1.59
90% Control	26.88 33.25	30.07	3.19

TABLE 4---SGPT Values for Guinea Pigs

Type of feeding trial	Hay formulation	Animal	SGPT (I.U.)	Mean	Standard deviation
Short-term	75% Experimental	GP1-E	24.31		
		GP2-E	18.56		
		GP3-E	25.59		
		GP4-E	21.75	22.55	2.69
	75% Control	GP5-C	31.99		
		GP6-C	14.08	23.04	8.96
Long-term	75% Experimental	GP7-E	31.35		
		GP8-E	28.15		
		GP9-E	17.92	25.81	5.73
	75% Control	GP11-C	16.00		
		GP13-C*	19.84	17.92	1.92

GP = Guinea Pig; E = Experimental; C = Control. * This animal was not sun-exposed.

TABLE 5---SGPT Values for Rabbits

Type of feeding trial	Hay formulation	Animal	SGPT (I.U.)	Mean	Standard deviation
Short-term	75% Experimental	R1-E	17.28		
		R2-E	14.08		
		R3-E	12.80		
		R4-E	17.92	15.52	2.14
	75% Control	R5-C	39.67		
		R6-C	26.23	32.95	6.72
Long-term	75% Experimental	R7-E	27.51		
		R8-E	27.51		
		R9-E	39.03	31.35	5.43
	75% Control	R11-C	37.11		
		R13-C	25.60	31.36	5.76

R = Rabbit; E = Experimental; C = Control.

TABLE 6---SGPT Values for Mice with CCl_4-Induced Liver Damage

Type of feeding trial	Hay formulation	SGPT (I.U.)	Mean	Standard deviation
Short-term	75% Experimental	13.44		
		28.80	21.12	7.68
	75% Control*	95.34	95.34	---
Long-term	75% Experimental	88.30		
		138.85	113.58	25.28
	75% Control*	69.10	69.10	---

* Control mice also received CCl_4.

DISCUSSION

The fungi isolated from the experimental hay were all common saprophytes and may not be representative of the flora present at the time of the photosensitization outbreak. During the year between the clinical outbreak and the initiation of these investigations, the hay was exposed to a wide variety of climatic conditions, and the fungal flora may have changed significantly.

Normal SGPT values for the mouse range from 9 ± 3[50] to 23 ± 8.6 I.U.[48] SGPT values from the short-term mice (Table 3) were essentially normal. The normal SGPT value for the guinea pig is 13 ± 2 I.U., and for the rabbit it is 14 ± 4 I.U.[50] The SGPT values from the experimental guinea pigs and rabbits (Tables 4 and 5) were comparatively high, but there was little difference between the experimental and control animals. The absence of liver lesions in any of these animals supports the SGPT values as being normal.

SGPT values in the mice injected once with CCl_4 (Table 6) were variable, with some values in the normal range and several levels greatly increased. However, the presence of histopathological lesions in the liver of all the injected mice, and the elevated SGPT values in mice given 2 injections of CCl_4 (Appendix X), confirmed that liver damage was present.

CCl_4 liver damage was produced in mice to determine if an increase in circulating plant or fungal pigments would

result with subsequent photosensitization. However, the liver damage produced might not have been of sufficient magnitude or the proper type to allow pigment accumulation.

It was unlikely that the long-term feeding trials were of too short a duration to allow lesions to develop. Lesions have occurred in guinea pigs eating facial eczema hay in as little as 10 days[39] and in rabbits in 7 days.[14] In the 1957-1958 outbreak of alfalfa hay disease, icterus appeared in cattle 10 to 12 days after they began consuming the toxic hay.[25]

The fact that the rabbits were able to maintain or increase their body weights and that the guinea pigs nearly maintained their body weights, with both experimental and control animals losing weight, indicated that neither an appetite depressant in the experimental hay not inadequate consumption of the hay was a significant factor. However, it may be possible that these animals did not consume enough of the hay's toxic agent to cause liver damage or photosensitization. Liver damage might have been produced if the hay had been extracted to concentrate the toxin.

The low chlorophyll in the diet should not have been a factor. Phylloerythrinemia can occur even in animals fed hay and chaff, since the pigment can be formed from chlorophyll degradation products in these materials. The level of blood phylloerythrin necessary to produce photosensitization probably is quite low, as it has been shown that

the level in sheep necessary to cause photosensitization may be less than 0.005 mg./100 ml.[13]

It is possible that the length of exposure to the sun may not have been long enough to produce skin lesions, although both the long-term guinea pigs and long-term rabbits were exposed on several subsequent days. Previous experiments in which mice and guinea pigs were photosensitized were conducted outdoors[17, 39] or the animals were sun-exposed daily for several weeks.[5]

An uneven distribution of the toxin in the hay may be another reason for lack of signs. It was noted in the 1957-1958 outbreak that hay from different areas of the same field varied considerably in the ability to produce the disease.[25]

It is likely that the toxic factor in the experimental hay was reduced or destroyed during the year it was exposed to the weather. Although the hay involved in the 1957-1958 outbreak of alfalfa hay disease was still toxic after 2 to 3 years, it had been kept in barn storage.[25, 34] Facial eczema grass kept its toxicity for only 1 year with storage at -10 C.[39] Facial eczema toxin is unstable and is present in low concentrations, even in highly toxic samples of dried grass.[38] The search for the toxic substance in facial eczema was hampered by the fact that by the time sheep showed signs, the hay was no longer toxic.[13]

It is also possible that the animals did not become photosensitized due to species variability or susceptibility, such as a difference in the ability to alter chlorophyll to phylloerythrin. This fact could also explain the previous failure[34] to photosensitize experimental animals using toxic alfalfa hay.

In future outbreaks of photosensitization involving alfalfa hay, extensive mycological examination of the hay should be conducted immediately. The serum of photosensitized cattle should be examined for abnormal absorption bands. If found, absorption spectra of extracts from the isolated fungi should be determined and the wavelengths compared with that from the serum. The absorption bands of phylloerythrin, a known photosensitizer, should also be looked for in the serum. This technique has proved useful to identify photodynamic agents.[12] Future investigations also should minimize variables by using ruminant experimental animals, through selecting representative samples of the suspected hay, and by storing the hay under conditions of minimal chemical alteration. If a fungus were responsible for the syndrome, feeding fungal cultures or extracts of the culture to cattle and other experimental animals would be indicated.

The 1971 aflatoxin level of 1,704 ppb B_1 equivalent may have caused or contributed to the liver damage in that outbreak, although the 1972 level of 1,123 ppb B_1 equivalent did not produce liver damage in the experimental animals.

A level of 700 ppb is sufficient to produce liver lesions in cattle during chronic feeding trials.[22] Regretably, aflatoxin determinations were not available at the time of the 1957-1958 outbreak.

It should be remembered that alfalfa hay disease may be due to a toxic metabolite produced by the alfalfa hay under altered growth conditions. A combination of liver damage produced by the toxic metabolite and the aflatoxins or other mycotoxins may have caused the photosensitization. This could be an example of synergism, such as has occurred with the simultaneous feeding of rubratoxin and aflatoxin B_1 to rats. In that instance, signs of toxicity resulted which were not attributable to either compound alone.[18]

SUMMARY

Alfalfa hay from an outbreak of hepatogenous photosensitization in cattle was fed to mice, guinea pigs, and rabbits in an attempt to reproduce the photosensitization in these species. Liver damage was induced with carbon tetrachloride in some of the mice to permit a biological increase in any photodynamic agent present in the hay. Photosensitization did not occur in any of the experimental animals. As measured by histological studies and SGPT levels, liver damage was not produced by consumption of the hay. The absence of photosensitization and liver damage may be due to low concentrations of toxin(s) in the hay, uneven distribution of toxin(s) in the hay, destruction of toxin(s) due to age and weathering, insufficient length of sun exposure, or species variability. Aflatoxins, or other mycotoxins in the hay, may have produced or contributed to the liver damage observed in the original photosensitization in cattle, although it did not produce liver damage in the experimental animals.

APPENDIX I---Daily Weights (Grams) of Short-Term Rabbits

Date	Animal					
	R1-E	R2-E	R3-E	R4-E	R5-C	R6-C
May 25	272	415	378	403	722	877
26	266	387	548	398	734	871
27	274	432	568	422	791	880
28	287	417	591	445	789	891
29	294	443	592	459	791	898
30	257	443	618	461	800	878
31	271	465	619	484	820	900
June 1	280	471	621	486	831	895
2	294	487	654	517	855	948

R = Rabbit; E = Experimental; C = Control.

APPENDIX II---Daily Weights (Grams) of Long-Term Rabbits

Date	Animal					
	R7-E	R8-E	R9-E	R10-E	R11-C	R12-C
May 25	481	640	823	517	814	905
26	503	614	763	453	755	860
27	506	615	775	500	821	891
28	523	651	795	514	811	914
29	509	630	788	517	807	910
30	510	640	800	518	806	910
31	534	658	804	558	841	917
June 1	548	687	826	574	857	943
2	522	725	902	611	892	985
3	533	699	849	591	875	943
4	527	713	886	589	911	977
5	560	744	957	604	951	986
6	590	726	936	633	935	985
7	594	720	914	626	930	994
8	663	763	948	691	978	1030
9	630	745	938	656	982	1027
10	733	838	1001	751	1089	1143
11	710	826	974	703	1058	1142
12	695	810	940	676	1035	1116
13	712	824	973	660*	1171	1229
14	724	840	994	-	979	1057
15	794	888	1055	-	1073	1119
16	758	892	1070	-	1105	1157
17	755	904	1089	-	1263	1272
18	724	931	1114	-	1267	1298
19	693	907	1072	-	1141	1174
20	720	910	1053	-	1022	1190
21	765	924	1045	-	1100	1222
22	758	946	1104	-	1126	1243
23	778	950	1128	-	1152	1200
24	823	958	1146	-	1220	1245
25	790	962	1116	-	1196	1261
26	816	952	1158	-	1197	1231
27	807	954	1147	-	1219	1245
28	832	946	1132	-	1233	1237
29	873	982	1173	-	1241	1243

R = Rabbit; E = Experimental; C = Control. * Died prior to termination of experiment.

APPENDIX III---Daily Weights (Grams) of Short-Term Guinea Pigs

Date	Animal					
	GP1-E	GP2-E	GP3-E	GP4-E	GP5-C	GP6-C
May 16	286	322	290	276	256	260
17	286	330	294	264	255	241
18	276	328	272	244	266	257
19	288	330	270	244	270	254
20	280	324	248	236	260	248
21	280	328	228	237	265	248
22	268	322	200	240	256	240
23	283	335	194	245	252	236

GP = Guinea Pig; E = Experimental; C = Control.

APPENDIX IV---Daily Weights (Grams) of Long-Term Guinea Pigs

Date	Animal						
	GP7-E	GP8-E	GP9-E	GP10-E	GP11-C	GP12-C	GP13-C*
May 16	274	254	262	282	176	237	282
17	280	272	276	282	180	220	275
18	289	254	278	278	194	228	281
19	290	268	278	282	190	225	278
20	272	248	266	276	184	228	282
21	273	243	262	272	182	227	274
22	264	234	256	262	180	226	282
23	260	236	244	269	184	229	278
24	254	249	234	271	186	228	286
25	253	244	245	270	180	233	290
26	260	242	227	258	187	234	292
27	262	244	230	244	186	233	296
28	269	240	230	234	186	230	295
29	258	242	230	214	188	228	286
30	260	242	238	203**	185	228	288
31	265	242	240	-	178	228	304
June 1	262	239	248	-	188	228	300
2	265	235	246	-	186	231	292
3	264	234	242	-	188	236	288
4	262	229	251	-	187	243	290
5	276	237	264	-	188	245	292
6	277	228	250	-	192	230	304
7	285	215	244	-	191	230	296
8	284	218	250	-	194	223	296
9	286	212	249	-	196	226	300
10	280	210	252	-	194	212**	306
11	276	200	240	-	208	-	302
12	280	212	236	-	206	-	302
13	279	215	247	-	205	-	292
14	265	221	238	-	200	-	294
15	268	221	238	-	191	-	298
16	280	224	239	-	196	-	284
17	265	220	234	-	188**	-	264
18	265	220	229	-	-	-	269

GP = Guinea Pig; E = Experimental; C = Control. * This animal was not sun-exposed. ** Died prior to termination of experiment.

APPENDIX V---SGPT Values for Long-Term Mice with CCl_4-Induced Liver Damage (Preliminary Experiment)*

Hay formulation	SGPT (I.U.)	Mean	Standard deviation
25% Experimental	34.55 39.67 43.51 58.87	44.15	9.07
50% Experimental	44.15 186.19	115.17	71.02
50% Control**	60.79 85.74	73.27	12.48

* Due to bad weather, the mice could not be sun-exposed 2 days after CCl_4 injection. They were given a second injection 17 days after the first. During sun exposure 2 days later, all but 8 of them died from heat prostration. The experiment was then repeated. ** Control mice also received CCl_4.

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INVESTIGATIONS IN LABORATORY ANIMALS OF A BOVINE PHOTOSENSITIZATION SYNDROME ASSOCIATED WITH THE CONSUMPTION OF MOLDY ALFALFA HAY

by

NANCY N. SCHARDING

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ABSTRACT

In the winter of 1971, hepatogenous photosensitization occurred in Kansas. It affected a herd of Shorthorn cattle fed silage and visibly moldy alfalfa hay. Aflatoxin or other mycotoxins present in the hay may have produced the preliminary liver damage necessary for hepatogenous photosensitization to develop. Similar field conditions in cattle and other domestic animals have been associated with alfalfa hay or moldy alfalfa hay, and with other legumes. Fungi or their toxins were suspected to have been the etiological agent.

Facial eczema of New Zealand sheep has been shown to be caused by a mycotoxin produced by the fungus Pithomyces chartarum growing on pasture grass. The fungus responsible for facial eczema has also been isolated from several areas of the United States. A photosensitization syndrome in the United States involving Bermuda grass also is thought to be a mold toxicity. Experimental photosensitization has been produced in mice by feeding cultures of Penicillium viridicatum. Circulating pigments produced by the fungus were suspected as the photodynamic agent.

In order to understand the causative factors associated with the 1971 Kansas hepatogenous photosensitization syndrome, studies were conducted in laboratory animals in an attempt to reproduce the condition and to define the probable etiologies. The alfalfa hay associated with the Kansas

photosensitization was fed to mice, guinea pigs, and rabbits for 1 or 4 weeks. The experimental feed was assayed for fungal flora and aflatoxin concentrations. Body weights of the experimental animals, SGPT levels, and liver histopathology were determined during and/or following the experimental periods. Liver damage was induced in some of the mice with carbon tetrachloride to permit a biological accumulation of any photodynamic agent present in the hay. The animals were exposed to direct sunlight for 2 hours to determine if they would become photosensitized.

The procedures used did not result in reproduction of the clinical hepatogenous photosensitization syndrome. SGPT values and histological exam of the livers from non-carbon tetrachloride treated animals were normal. Photosensitization did not develop in any of the experimental animals.

The lack of liver damage and the absence of photosensitization may have resulted from a low concentration of toxin(s) in the hay, uneven distribution of toxin(s) in the hay, destruction of the toxin(s) due to hay deterioration and weathering, insufficient length of sun exposure, or by species variability in the experimental animals. Future investigations should minimize variables by using ruminant experimental animals, through selecting representative samples of the suspected hay, and by storing the hay under conditions of minimal chemical alteration.