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A Study Of The Toxic Principle in Red Clover

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ABSTRACT

Second-cutting red clover hay occasionally contains a toxic factor which causes cattle and sheep to slobber and go off feed. Other symptoms of toxicity such as diarrhea, bloat, stiff joints and even death have been observed on Missouri farms.

In recent years an increasing number of cases of toxicity have come to the attention of the Experiment Station and an attempt has been made to identify the causative agent. A knowledge of its chemical nature should prove helpful in determining the reason for its occurrence and how to prevent it. A guinea pig assay for the toxic factor has been developed and used to aid in detection of toxic hay.

Although hay cut from small grain stubble is the most common source of trouble, the toxin is associated with the red clover portion. The toxic hays do not contain a high concentration of saponins, as estimated by the usual saponin tests. The toxic principle is soluble in, and can be extracted from the hay with, water, ethyl alcohol, and chloroform. It is organic in nature and it slowly loses activity while in solution.

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A Study Of The Toxic Principle in Red Clover

INTRODUCTION

During the past ten years numerous samples of red clover hay have been submitted to the Experiment Station for testing because the hay had caused slobbering and symptoms of toxicity in cattle and other livestock. The number of samples sent to the Station indicates that the problem is wide spread in this state and personal communications have indicated the same difficulty exists in other Midwest states, including Illinois, Indiana, and Ohio.

In all cases brought to our attention the hay was either cut in the fall from stubble after spring seeding or was second-cutting hay. The fact that second-cutting red clover hay frequently causes excess salivation in livestock has been recognized for many years, but nothing is known about the chemical nature of the causative agent nor the conditions that give rise to it.

A laboratory method has been developed for the assay of the toxic principle; preliminary procedures for its isolation are described.

MATERIALS AND METHODS

Hay Samples—The samples studied were submitted either by a county agent or a farmer whose animals experienced difficulty. A brief description of the samples examined is given in Table 1. Note that all samples were reported to cause slobbering. The other symptoms were variable and differed in intensity.

In most cases the animals refused the hay after the first feeding unless forced by starvation. That the hays caused excessive salivation was confirmed by the use of the guinea pig in all cases except the one lespedeza-grass sample, number 5458.

Guinea Pig Assay—The basal ration was a stock colony ration either identical with or similar to ration 16, described by Hogan, House, and Regan (1954). The hay was finely ground in a hammer mill and in most cases it was mix-

TABLE 1--DESCRIPTION OF HAY SAMPLES

Laboratory Number	Season Grown	County Where Grown	Type of Hay	Symptoms Reported
2496	1949	Marion	Red clover-lespedeza mixed with stubble	Cattle slobbered profusely; refused hay.
2802	1950	Monroe	Second-cutting red clover	Cattle and horses slobbered and refused feed.
2804	1950	Carroll	Second-cutting of red clover with considerable grass	Cattle and horses slobbered profusely
2810	1950	Dade	Red clover mixed with grass, cut from spring seeding	Cattle slobbered, bloated and became stiff in the joints.
2870	1950	Schuyler	Second-cutting red clover with very little grass	Cattle slobbered and sheep became sick
3068	1951	Jasper	Red clover with stubble. Spring seeding with oats.	Cattle slobbered after one feeding, then refused hay.
3135	1951	Ray	Red clover, grass and wheat stubble	Sheep slobbered
3136	1951	Randolph	Red clover and wheat stubble	Cattle, horses and sheep slobbered. Mare became sick and aborted.
4303	1955	Chariton	Red clover, wheat stubble	Cattle slobbered and refused feed.
4453	1955	Calloway	Second-cutting red clover	Slobbering in cattle but most marked in calves and sheep.
4465	1955	Oregon	Silage from second-cutting red clover	Cattle slobbered and refused feed.
4790	1955	Shelby	Second-cutting red clover	Dairy cattle slobbered and milk production dropped.
5459	1957	Livingston	Red clover from spring seeding cut in fall	Cattle slobbered and had diarrhea. One died.
5457	1958	Nodaway	Red clover stubble	Cattle slobbered profusely after one feeding. Lost weight.
5458	1958	Lincoln	Lespedeza-grass	Dairy cattle slobbered, (Not confirmed in guinea pigs).

ed with an equal weight of ration 16. In some trials the hay replaced alfalfa meal in the basal ration.

Guinea pigs ranging from 200 to 800 grams in weight were fed the mixture for at least one week and in some trials for as long as four weeks. The animals were weighed at intervals of one to three days and examined daily for excessive salivation. As an index of the degree of salivation, the response was graded 1+ for slight, 2+ for intermediate and 3+ for the most severe degree.

Animals which graded 3+ drooled so much that the saliva kept the fur on the throat wet. Eventually the fur on these soaked areas came out and a dermatitis developed. An animal that graded 3+ is shown in Figure 1.

The sensitivity of a group of animals to a given sample of hay varied from slight to severe but virtually all animals fed the hays slobbered to some extent. An animal was examined by holding it vertically in both hands and opening its mouth with the thumbs. Under these conditions the mouth of a guinea pig that has consumed toxic hay will fill with saliva and the saliva may even flow out.

Preparation of Extract for Saponin Test—Three different methods of extraction were tested. These were the method of Peterson (1950), that of Burrell and Houston (1948), and that of Kofler (1927). The three methods gave similar results so the direct method of Kofler, which was simplest, was used more extensively. In this method 1 gram of ground hay was added to 10 ml. of M/15 K_2HPO_4 solution and allowed to stand at room temperature for 5 hours. The suspension was then heated for 30 minutes at 80° C and centrifuged. This supernatant liquid was used for the saponin assay.

Determination of Hemolytic Index as a Measure of Saponin Content—The method of Kofler (1927) was used. A 2 percent suspension of bovine red cells was prepared from citrated blood by washing eight times with isotonic saline solution. For the test, 1 ml. of the red cell suspension, 3 ml. of a mixture of equal parts isotonic saline and M/15 K_2HPO_4, and 1 ml. of hay extract were added to a test tube in the order named. After these ingredients were mixed, they were allowed to stand in the tube for 20 hours. For preparation of a standard curve, purified saponin (Merck) solutions (0.002 to 0.02 percent) were substituted for the hay extract. All solutions were read against a reagent blank set at 100 percent transmittance with a 520 filter. Blanks for correction due to the color of the hay extracts were prepared by substituting saline solution for the red cell suspension. The absorbances of the blanks were subtracted from the absorbance of the test system to give an estimate of the amount of hemolysis. It should be noted that these blanks had high absorbance in all cases and varied between 0.3 and 0.4.

Foam Test for Estimation of Saponin Content—In this test, 5 ml. of hay extract were shaken in a 10 ml. graduated cylinder for 1 minute. The amount of foam was measured immediately and at intervals thereafter until the foam volume was reduced to 10 percent of the original. It was decided that stability of the foam, i.e. time required for foam to reach 10 percent of its original volume,

was a more reliable index of saponin content than the total foam produced.

Determination of Surface Tension—Surface tension was measured with the DuNouy tensiometer. The extract was placed on a watch glass, the tensiometer ring immersed, and the reading made immediately. Readings made after the ring had been immersed 10 minutes were somewhat lower but were not considered to be more reliable as a measure of saponin content.

Preparation of Hay Extracts for Feeding Experiments—

(a) Aqueous Extraction—Five kilograms of ground hay were placed in 80 liters of water and the suspension heated to boiling. The coarse particles were removed by straining through muslin and the finer particles by filtration through paper. The filtrate was concentrated *in vacuo* to about 20 percent dry matter. The yield from hay No. 4303 was 730 grams of dry matter.

(b) Ethyl Alcohol Extraction—One kilogram of ground hay was heated under reflux with 7 liters of 95 percent ethanol for 1 hour. After it had been cooled the extract was decanted and the residue extracted twice more with 5 liters of alcohol each time. The combined extracts were evaporated *in vacuo* to about 12 percent dry matter. The yield from hay Number 4303 was 116 grams.

RESULTS

Response of Large Animals—To confirm reports that the hay under study caused slobbering in large animals, one sample, number 2496, was obtained in quantity and fed to cattle and sheep. Eight Hereford heifers were taken off pasture and placed in dry lot. They were fed for a period of 10 days a ration consisting of 10 pounds of the hay and a concentrate of corn and bran. They first showed signs of excess salivation on the third day but continued to eat the hay and to slobber throughout the period.

Three yearling ewes were confined for a period of 2 weeks and fed daily 8 pounds of hay and 1 pound of mixed corn and oats. They ate all of the clover and lespedeza in the sample and left the stubble. They showed no signs of slobbering at any time and no change in weight.

Response of Guinea Pigs—Guinea pigs fed a ration composed of equal parts of a practical colony ration and toxic hay usually showed symptoms of excess salivation within 48 hours and sometimes within 24 hours. The severity of slobbering became more marked and reached a peak after 5 days unless the animals refused to eat. The amount of saliva produced varied during the day and was greatest immediately after eating. As might be expected the toxicities of the hays varied widely. Some hays caused marked weight loss and eventually death in addition to salivation; others caused only mild growth rate depression and slobbering.

Examples which illustrate the response of animals to various toxic hays are shown in Table 2. For comparative purposes, a red clover control hay, number 3083, which had been fed to Experiment Station livestock without difficulty,

TABLE 2--RESPONSE OF GUINEA PIGS FED TOXIC HAY, A CONTROL HAY AND SAPONIN

Number of Hay	Composition of Ration Fed	Initial Weight	Duration of Test	Average Daily Feed Consumption	Average Daily Weight Change	Degree of Salivation	Survival Period
		grams	days	grams	grams		days
3083	50 parts R16	195	10	--	+ 0.5	0	--
Control	50 parts hay	195	10	--	+ 2.5	0	--
		245	7	28	+ 4.3	0	--
3083 + Saponin	50 parts R16 50 parts hay 0.1% saponin	195[1]	21	--	+ 2.6	0	--
		245[2]	10	--	+ 4.5	0	--
3068	50 parts R16	185	10	--	- 1.0	+++	10
	50 parts hay	200	4	--	- 7.0	+++	4
		220	7	20	- 7.0	+++	7
		275	10	17	- 4.8	+++	--
4303	34 parts hay	677	28	--	- 9.5	++	--
	Sub. for al-	585	13	--	- 8.5	+++	13
	falfa in R16	707	14	--	- 5.7	+++	--[3]
		535	16	36	-12.0	+++	16
5457	50 parts R16	544	14	--	0.0	+++	--
	50 parts hay	615	14	--	0.0	+	--
		306	14	--	+ 3.0	++	--
		305	14	--	+ 0.7	+	--

1. This animal received 0.1% of a commercial product labeled "Saponin".
2. This animal received 0.1% of a commercial product labeled "Toxic Saponin".
3. Animal sacrificed for pathological examination.

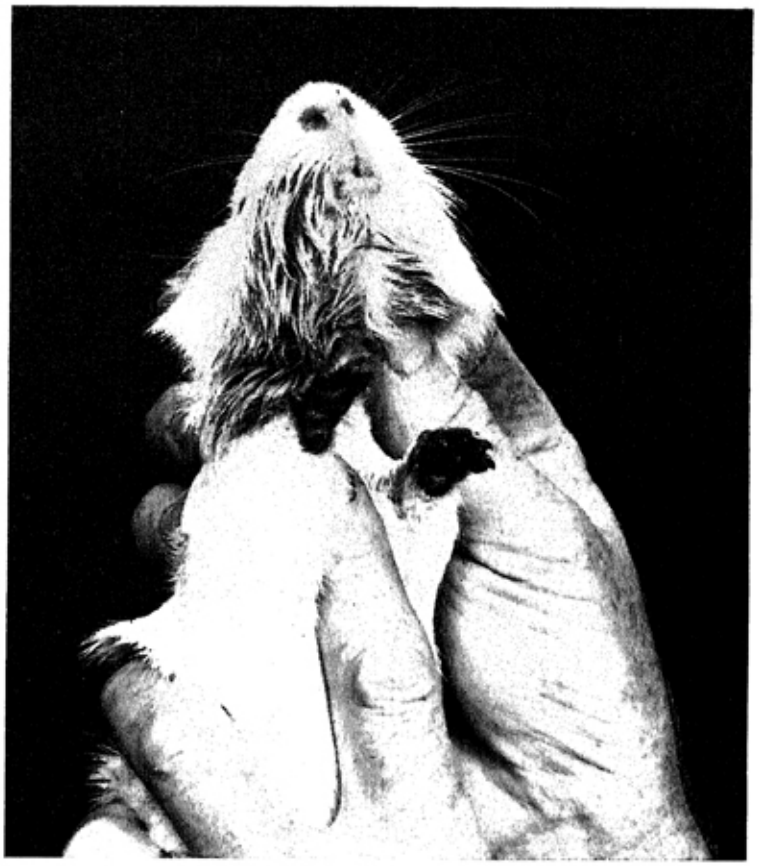

FIG. 1—THIS GUINEA PIG HAS CONSUMED A TOXIC HAY FOR 10 DAYS. NOTE THAT THE FUR IS WET FROM CONTINUOUS DROOLING OF SALIVA. THIS IS THE POSITION IN WHICH ANIMALS WERE HELD FOR EXAMINATION.

was fed to three guinea pigs. These animals gained weight and showed no signs of slobbering.

The addition of 0.1 percent of two different commercial preparations of saponin, one of which was labeled toxic, produced no adverse symptoms.

Hay sample 3068 caused marked weight loss and slobbering and three of four animals succumbed within ten days. Sample number 4303 was as toxic but when fed several months later showed less toxicity. This and other examples indicate that the hay may lose toxicity upon prolonged storage, especially if ground.

Other samples, such as number 5457, produced excess salivation but did not cause weight loss or death. Animals that consumed the more severely toxic hays consumed more water than normal and frequently showed enlarged lymph nodes and general dehydration of tissues as exemplified by a small spleen and liver. Hemorrhage in the urinary tract and intestines were observed occasionally. Histological examination confirmed the gross appearance of dehydration and indicated hyper-activity of the salivary glands.

Oral administration of aqueous and alcoholic extracts of toxic hay produced excess salivation in animals within 30 minutes to 1 hour and the response persisted for at least 4 hours. Intraperitoneal injection of purified extracts produced peripheral dilation of blood vessels and contraction of throat and abdominal muscles, but no salivation. It is not known whether or not the response observed after injection of extracts is caused by the same principle that causes excess salivation after oral treatment.

Tests for Saponin in Toxic Hay—The fact that the most common symptom of toxicity was slobbering suggested that saponins were the causative agent. Saponins are common plant constituents which have a soap-like action in lowering surface tension. Kofler (1927) stated that ingestion of saponins caused salivation and Ewart (1931) reported that several livestock diseases might be due to prolonged ingestion of saponins. The Kimberly Walking Horse disease was found to be the result of long term ingestion of "white wood" which contained 2 to 8 percent of saponin.

Three different tests were used to estimate the saponin content of hay samples under study. One was based on hemolysis of red cells and would be useful only for the detection of hemolytic saponins. Another was based on foam stability and the third on the lowering of surface tension.

Figure 2 shows the standard curves that relate absorbance after hemolysis, and foam stability, to concentration of pure saponin. There was a straight line relationship between absorbance of the hemolyzed cells and saponin concentration over the range from 0.002 to 0.01 percent of saponin. The graph relating foam stability and saponin concentration was also a straight line from 0.02 to 0.05 percent.

In some cases the clover was separated from the grass and stubble mechanically and extracts were prepared from each of the two fractions. In other cases the total sample was extracted and the extract subjected to the three tests. The results are shown in Table 3. The estimated values are not to be considered abso-

TABLE 3--ESTIMATED SAPONIN CONTENT OF HAY

Hay No. and Fraction	Hemolytic Test		Foam Stability		Surface Tension DuNouy Tensiometer
	Corrected Absorbance	Estimated Saponin Content	Time to Reach 10% Volume	Estimated Saponin Content	
		%	Min.	%	Dynes per cm.
3083 clover (control)	0.08	0.02	15	0.05	45.6
2496 clover	0.10	0.025	8	0.025	50.4
2496 grass	0.20	0.048	300	0.33	45.5
2802 clover	0.08	0.02	120	0.19	42.2
2802 grass	0.41	0.10	45	0.12	45.6
2804 mixed	0.07	0.02	480	0.45	44.7
2810 mixed	0.32	0.08	45	0.12	45.1
2870 clover	0.09	0.02	15	0.05	47.6
3068 clover	0.10	0.025	5	0.01	51.7

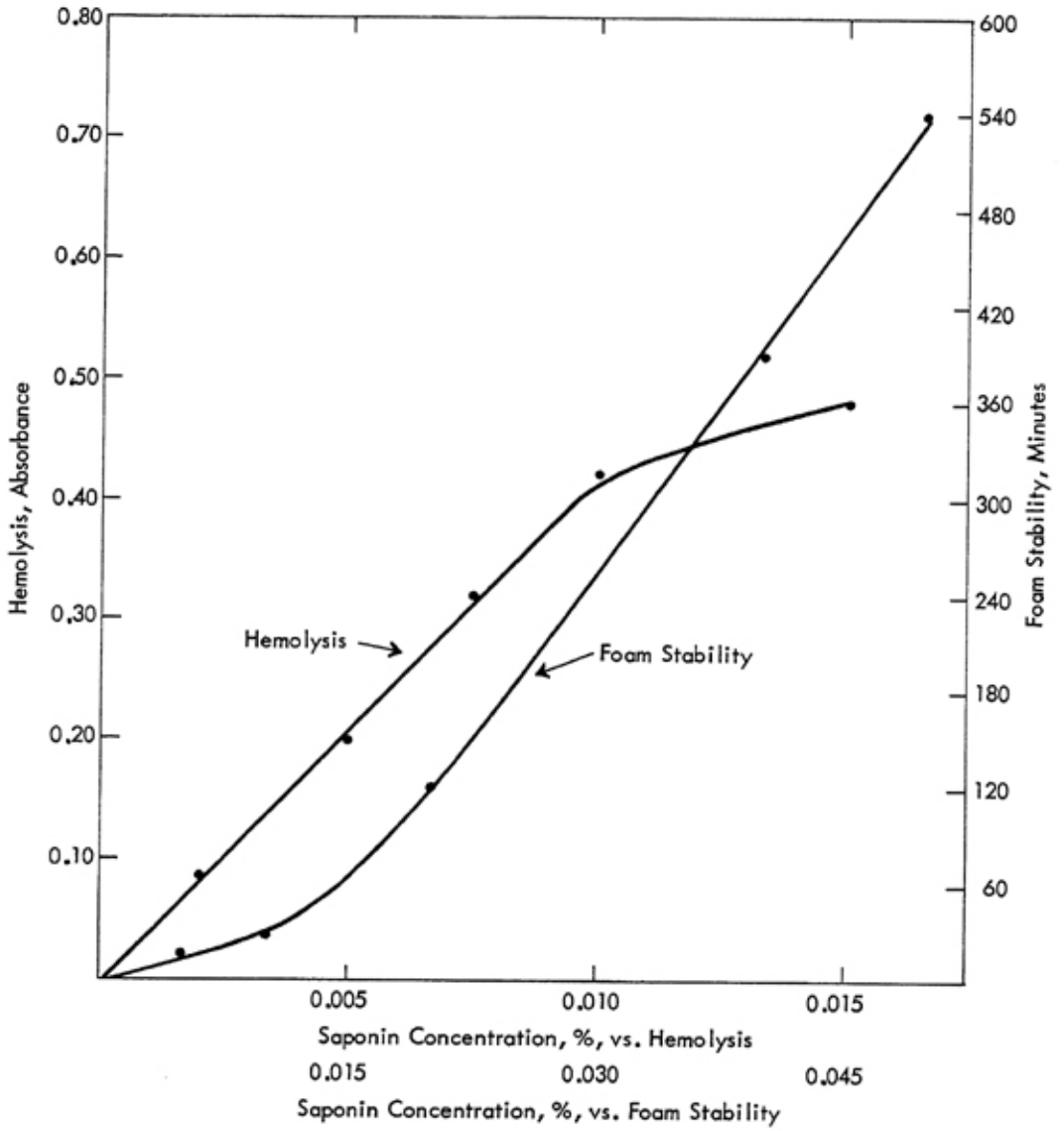

FIG. 2—STANDARD CURVES RELATING HEMOLYSIS (ABSORBANCE) AND FOAM STABILITY TO SAPONIN CONCENTRATION.

lute in any sense but they are useful indices for comparative purposes. Other data to be presented show that the toxicity is associated with red clover and not with the grass or stubble, yet in many cases the estimated saponin content of the grass was higher than that of the clover. Furthermore, in most cases the toxic hays contained no more saponin than the control hay. These results led to the conclusion that there was no relationship between the saponin content as measured by the methods used and the toxicity of the hay.

Mechanical and Chemical Fractionation of Toxic Hay—Hay No. 4303 was one of the most toxic samples tested. Since it was available in bale quantity, it was fractionated in an attempt to identify the causative agent. A summary of the fractions prepared and their biopotency is in Table 4.

TABLE 4--MECHANICAL AND CHEMICAL FRACTIONS PREPARED FROM HAY NO. 4303

Ration Number	Description of Fraction	Amount Fed Hay or Equiv.	Guinea Pig Assay	
			No. of Animals	Response
		%		
4304	Original mixed hay, 4303	34	4	+
4668	Straw from 4303	35	2	-
4669	Weeds from 4303	35	2	-
4670	Red clover stems from 4303	35	2	1+
				1-
4671	Red clover leaves from 4303	35	2	+
	Original hay, 4303	15	2	1+
				1-
4584	Ash of hay 4303	35	2	-
4466	Aqueous extract of 4303	35	7	+
4655	Aqueous extract of 4303	5 D.M.(a)	2	+
4662	Aqueous extract of 4303	2.5 D.M.	2	+
4673	Aqueous extract of 4303	1.0 D.M.	2	+
4337	Ethanol extract of 4303	30	1	+
4310	Residue after ethanol extract	30	1	-
4356	Chloroform soluble portion of solids extracted by ethanol	30	2	+
4652	Chloroform extract of 4303 after petroleum ether extraction	30	2	+
4627	Chloroform soluble portion after partitioning with an aqueous extract at pH 3	30	1	-
4625	Chloroform insoluble residue from 4627	30	2	+
4641	Chloroform soluble portion after partitioning with an aqueous extract at pH 10	30	2	-

(a) D.M. refers to grams of dry matter of the fraction fed while other values in the column refer to hay equivalents.

The hay was first separated mechanically into straw, weeds, clover stems and clover leaves. Most of the toxic principle was found in the clover leaves with a lower concentration in the stems. When fed as 34 percent of the total diet, the mixed hay caused excess salivation in all test animals; at 15 percent it showed only borderline activity. Ash prepared from the hay was inactive.

A hot water extract of the hay was highly active and caused slobbering when fed at levels as low as 1 percent dry matter in the diet. Hot ethyl alcohol also extracted the toxin and left no appreciable activity in the residue.

From the standpoint of concentrating the factor, alcohol extraction seems to offer more promise than aqueous extraction. When the alcohol was removed

in vacuo the principle could be solubilized by refluxing with chloroform. It could also be extracted directly from the hay by chloroform after a preliminary extraction with petroleum ether. However, when an attempt was made to partition the toxin between chloroform and an aqueous solution at pH 3 or pH 10, no activity could be detected in the chloroform layer. Thus, it was judged to be infinitely more soluble in water than in chloroform. Neither could the factor be extracted into ethyl acetate from aqueous solution. Considerable inert material was removed by a process which involved dissolving in butanol the solids remaining after removal of the alcohol from an extract. The solution was then partitioned with water and all of the activity went into the aqueous phase while considerable inert material remained in the butanol.

The chemical nature of the toxic factor cannot be deduced from the information at hand, but its properties are similar in many respects to those of alkaloids. It was observed that all fractions gradually lost activity upon standing or during laboratory manipulation. This loss of potency might be the result of oxidation in as much as many alkaloids are easily oxidized.

Attempts to Alleviate Toxicity—Various attempts have been made to eliminate the toxin or to counteract the symptoms but with little or no success. Toxic hay has been fed with high levels of the B vitamins, with a high level of ascorbic acid, and with 5 percent molasses without benefit. Since there was evidence that the factor might be readily destroyed by oxidation, permanganate was added to the drinking water (1:3000) in hopes of oxidizing it in the intestinal tract. This treatment did not prevent slobbering but it tended to increase feed consumption and the rate of gain. The possible beneficial effect of permanganate for guinea pigs consuming toxic red clover offers promise but needs confirmation.

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