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STUDIES ON CHANGES IN VITAMIN CONTENT OF ALFALFA HAY

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and C. E. Vail

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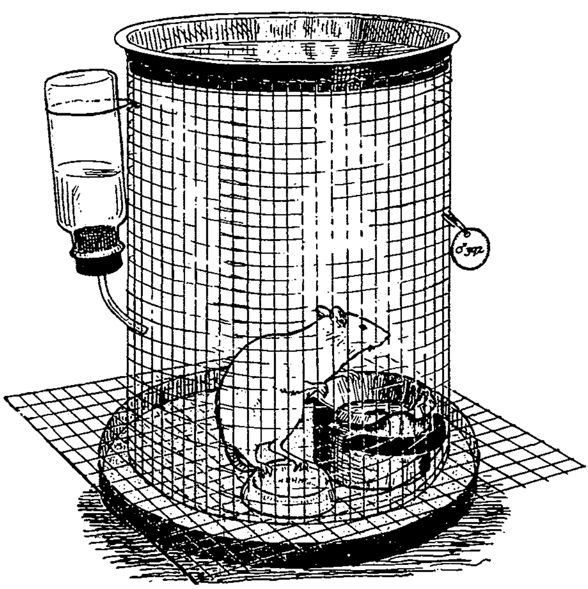
Part I.—TECHNIQUE USED

Since the technique of vitamin determinations is not standardized to the extent that chemical methods have been, we publish our methods somewhat in detail to answer the questions which immediately arise as to how we determined the vitamins and what standards were used.

The methods used for determining any single vitamin were the quantitative ones developed by Sherman, et al, based on keeping the rats gaining, after a depletion period, solely thru feeding the lowest adequate level of the food being assayed. All other vitamins and food constituents were adequately supplied.

The Equipment

A description of the equipment used and of the feeding technique may be found in the Journal of Home Economics.*



Type of individual cages and equipment used for experimental rats.

The Stock

The stock consisted of albino and black and white rats obtained from several sources of good parentage, mostly Wistar. The albino and black and white strains were kept separate.

The stock diet was composed of two-thirds whole wheat, one-third whole milk and 2 and one-half percent sodium chloride, but as our source of whole milk did not supply enough vitamin A for reproduction and lactation, this diet was modified to include a leaf of green lettuce once a week. Six grams of lean beef were supplied three times a week. This diet kept the stock healthy but gave the young rats only a moderate store of vitamin A.

*Vol. 23, p. 45 (1931).

The above diet limits the amount of vitamin A by the amount of butterfat in the whole milk and the lettuce. Vitamins B, G, E and D are supplied in abundance by the wheat, whole milk and lettuce.

In breeding the rats, one male is placed in a stock cage with three females and vaginal smears taken to catch the appearance of sperm cells in the vagina. When sperm cells appear, which is usually the case when cornification of the cells occurs, the female is removed and the time when the expected litter is to appear is marked on a record card kept for each rat. The time of gestation is 21 to 22 days.

Four or five days before the litter is expected, the female is weighed daily to find out whether the fetus is gaining normally or resorption is taking place. If too many litters are being lost thru resorption or being too weak to live, or if lactation is not proceeding normally, the diet must be modified. Sometimes a gram of yeast for a day or two will increase the milk flow. But usually the mothers must have an increase in vitamin A which is accomplished as noted above by increasing the amount of lettuce.

The pans and food cups were cleaned and sterilized once a week, the water bottles every 2 weeks.

The albino or black and white rats used in this investigation were 28 or 29 days old when they were weaned, weighed, marked and placed in individual round cages with screen bottoms to prevent access to excreta. At this time the rats weighed 40 to 55 grams and those over or under these weights were usually rejected.

The food cup always had a supply of the basal diet, the food being weighed in as needed, and that which remained being weighed back, thus keeping a record of the food consumed. Distilled water was always available.

The lower pan containing the paper towel was cleaned three times a week, any food scattered on the towel being screened out and returned to the food cup. A clean animal laboratory was maintained at all times.

VITAMIN C

The vitamin C or anti-scorbutic factor cannot be determined with rats as they are nearly immune and only with difficulty show signs of scurvy. Parsons came to the conclusion that "The rat produces vitamin C in metabolism from some source not available to the guinea pig." The guinea pig is used to determine vitamin C since, on a scorbutic diet, scurvy is produced in a short time.

In making the vitamin-C determinations, we had trouble with the basal diets. While scurvy was always produced, the guinea pigs would not eat the diet and many intestinal disorders complicated the scurvy symptoms. Sometimes the question arose whether death was due to starvation or scurvy.

Coen and Mendel devised a ration in the form of a soybean cracker with the following composition:

	Grams
Calcium lactate	15
Sodium chloride	15
Yeast powder	15
Butter	25
Skimmilk powder (heated 2 hrs. at 110° C.)	45
Soybean flour (cooked 30 min. 15 lbs. steam pressure)	385

This diet produced no bloating, congestion or other apparent alimentary disorders, yet scurvy was always produced.

Sherman, La Mer and Campbell's (19) standard vitamin-C free diet also gave excellent results. Its composition is as follows:

	Percentage
Ground oats	59
Skimmilk powder (heated to 110° C. for 3 hrs.)	30
Butter fat (melted and freed from salt and buttermilk)	10
Sodium chloride	1

VITAMIN-A TECHNIQUE

THE STANDARD TEST ANIMALS.—The standard rats used in this work, together with the equipment and method of handling, have been discussed. A point to be emphasized is the necessity for controlling the diet fed to the mother rats so that the young rats at weaning shall not have too great a body store of vitamin A.

THE DEPLETION PERIOD.—As is well known, in using the growing rat for the determination of vitamin A, it must be depleted of its body store of this vitamin before the experiment or determination can begin. This depletion period must not be too extended as the sensitivity or response of the animal becomes less as it grows older. For this reason the weight limits for most of the rats used in the vitamin-A work were placed at 40 and 55 grams, at weaning. Our experience has shown that, as a rule, if the weight at weaning is below 40 grams, the rat is likely to be too weak, with an abnormally short depletion period and the end of depletion too difficult to determine. If above 55 grams, the rat might prove to be too strong, and to have too long a depletion period. Some rats that had acceptable weights at weaning weighed 140 grams or more, at or before depletion. A few other rats failed to be depleted in 30 days. Such rats were considered to be too heavy, or to have too large a store of vitamin A, and were rejected.

The usual time required for depletion was between 3 and 4 weeks. The basal diet, described below, free of vitamin A, was fed the young rats while on depletion, as well as afterwards when the foodstuff being examined was fed. The weight records of the animals on depletion and their characteristic symptoms of vitamin-A deficiency are noticed further below.

DEPLETION RATIOS.—The length of time required for depletion, as well as the weights of the rats when depleted, showed some variation. We found, however, that the relation of the weight at weaning to the weight at depletion was a ratio which, on the average, fell within rather restricted limits. For example, a rat which weighed 48 grams at weaning and 96 grams at depletion was said to have a depletion ratio of 2.0. For the 625 rats used on the vitamin-A work for two seasons, the average weight at weaning (W) was 46.9 grams; the average weight at depletion (D) was 102.3 grams; the average depletion ratio (D/W) was thus 2.2. The extremes among the depletion ratios were 1.3 and 3.2.

From the foregoing discussion it may be concluded that most of our rats were not far from being average, normal, or standard rats.

THE BASAL DIET.—The experience of many workers on vitamins has shown that the basal diet for the experimental animals is of prime importance. It must be as free as possible of the vitamin which is to be determined, yet it must be capable of sustaining life and growth in a normal manner when the vitamin which is absent from such a diet is supplied to the animal. Experience has likewise shown quite accurately what the relative amounts of the various essential nutrients should be in basal diets.

For our determinations of vitamin A in alfalfa hay we have used our Diet 14, a modification of the one described by Sherman and Smith (19). Diet 14 had the following composition:

	Percentage
Extracted corn starch	75
Purified casein	18
Osborne and Mendel salts*	4
Sodium chloride	1
Agar-agar, powdered	2

The preparation of this diet, free of vitamin A, requires considerable care and thoroness. Using unextracted corn starch we found that our rats could not satisfactorily be depleted of their body store of vitamin A. Preliminary trials with dextrinized starch gave rather unsatisfactory results, principally because the rats refused the diet containing it, resulting in starvation.

For these reasons the corn starch was thoroly extracted with alcohol and ether before use in the basal diet for vitamin A.

The crude casein was purified by shaking with acidulated water, extracting with cold, then with boiling alcohol, and finally extracting twice with ether.

As to the agar-agar in Diet 14, our experience indicates that it is a beneficial ingredient, promoting digestion.

The basal diet being readily reproducible, we may expect that a given set of results obtained with its use may be paralleled or possibly duplicated at will when the other controlling factors, such as the standard test animals, are equal or comparable.

*J. Biol. Chem., 37:187.

While the ingredients of the basal diet may seem to have received rather drastic treatment in their preparation, the evidence supplied by the records of our positive controls, discussed later in Part II, indicates that Diet 14 was adequate in all respects, save vitamin A, for normal growth and well-being in rats of their age. That Diet 14 was A-free was amply demonstrated by our negative controls.

As supplements to the basal diet, all the vitamin-A rats were fed 0.5 gram dried yeast daily, to supply vitamins B and G, and were also irradiated with a sun-ray lamp for 20 minutes twice a week, to supply vitamin D.

Since vitamin C is not considered to be necessary to the rat, we did not attempt to provide this vitamin in the diet for our experimental rats. Likewise, we omitted fat from Diet 14 as the necessity of providing fat in the rat's diet seems to be an open question. Our experience indicates that the small amounts present in the alfalfa hays, or in the cod liver oil, were adequate.

THE WEIGHT RECORDS.—The rat-growth method of determining vitamins necessarily involves a weight record covering the normal period of most rapid growth, namely, the 8 to 12 weeks after weaning. The average rat, on a completely adequate diet, is not full-grown in that time, but later growth is not nearly so rapid and is likewise less profoundly affected by the lack of nutritional factors.

During depletion the animal was weighed twice a week, until near the end. The loss of the rat's store of vitamin A was indicated by a definite slackening in the rate of growth, down to the point where growth ceased. By weighing the rat daily as this point was approached, and also by noting any external symptoms of A deficiency, (discussed more fully below) we could usually find the exact point at which the rat was said to be depleted. The weights of the rat and of the basal diet remaining in the food cup were then recorded, and the rat placed on the experiment, i. e. it was fed a definite daily ration of hay in addition to Diet 14, plus yeast. Semi-weekly weighings of the rat were made thereafter until death or until the end of the 8-week period, when the weights of the rat and of the food remaining were taken. All these weighings gave the data from which the vitamin-A content of the hay and the weekly food consumption were calculated.

LEVELS AND UNITS.—The definite weight or ration of a given kind of foodstuff, as fed to a group of test animals, is referred to as a "level." A fuller account of the levels used in our hay investigations is to be found in Part II. The ideal level was considered to be one which would produce an average gain per rat of 3 grams per week, or 1 unit of vitamin A. This unit is defined on page 21.

SYMPTOMS OF VITAMIN-A DEFICIENCY.—During the experimental period, if the rat can get vitamin A from the alfalfa hay, it will continue to grow, but if insufficient vitamin A is present, a gain may be made for a time, but eventually a decline in weight starts in (growth ceases), accompanied by symptoms of lack of vitamin A.

The first of these symptoms, usually, is xerophthalmia which often appears at or near the end of the depletion period, first manifested by a reddening of the eyelids; incrustations then appear, sometimes the eyelids are sealed shut, and in the final stages, lesions or abscesses appear in the corners. The first cause of this series of changes seems to be the atrophy of the lachrymal glands. Since there are no tears to wash the eyeballs, the conjunctiva sack becomes irritated and conjunctivitis ensues.

Not all rats lacking vitamin A exhibit xerophthalmia. Other diseases often occurring are sinusitis, mastoiditis and abscesses in the sublingual and submaxillary glands. Respiratory troubles often develop, such as wheezing, gasping and tubercular conditions of the lungs.

One of the best symptoms is the condition of the adipose tissue in the abdomen and under the skin. Cramer makes a distinction between ordinary adipose tissue and glandular adipose tissue; and there seems to be a real difference, for the ordinary fat may disappear completely and the rat may still live, but when the adipose tissue disappears from around the kidneys, the neck, and between the shoulder blades, the rat seems unable to survive.

In the male rats one of the surest signs of lack of vitamin A is atrophy of the testes or the prostate gland.

In a number of cases the xerophthalmia, wheezing and even abscesses, apparent at depletion, disappeared entirely when the rats were given adequate rations of hay, or were used as positive controls.

AUTOPSIES.—All negative controls, and in fact all of the experimental rats that died, or were in obviously poor condition at the end of the 8-week period, were autopsied and records made of the findings. The object, of course, was to establish the cause of death, for abnormalities other than vitamin-A deficiency might cause death. In most cases it was readily seen that the animal suffered from the lack of vitamin A. The internal symptoms looked for at autopsy were essentially those outlined above, such as enlargement of, or pus in, the salivary glands; pus in sinuses or inner ears; absence of adipose and subcutaneous fatty tissue; digestive, excretory and reproductive organs abnormal in size, color or condition.

The autopsies also helped to corroborate the weight or growth curves. These two lines of evidence enabled us to state rather definitely whether or not a rat had been suffering from lack of vitamin A, and approximately to what extent. From such data we were enabled to make the necessary adjustments in the levels of hay fed.

VITAMIN-B TECHNIQUE

The experimental animals used in both the B and G studies, reported in this paper, were of the identical stock described above, up to the time of weaning. Many of the litters were split, some of the animals being used for A tests and others from the same family being taken for B or G tests as required.

BASAL B-FREE DIET.—After making preliminary tests, the diet finally selected for our B tests was a modification of Morgan and Bishop's formula, as follows:

	Percentage
Extracted casein	18
Commercial starch	49
Osborne and Mendel's salt mixture	4
Ground agar-agar	2
Lard	12
Autoclaved yeast powder	15

This was our Diet 4.

In addition to this diet, vitamins A and D were supplied in the form of 3 drops of cod liver oil, placed upon the daily hay ration, or simply in glass caster cups.

PREPARATION OF B-FREE CASEIN.—Any one of several grades of commercial casein was found satisfactory for our Diet 4 after it had been subjected to the process of purification by repeated extraction, first with water and then with alcohol.

Altho the regular commercial starch used in our studies was found to contain appreciable amounts of vitamin A, none was found which contained deleterious amounts of B, and it was unnecessary for us to extract the starch before using for B or G tests.

A bacteriological grade of ground agar was used in preparation of our B-free Diet 4, principally to supply some roughage, and was found to be helpful in preventing alimentary disturbances in the animals.

Practically all standard grades of lard as they appear on the market were found adequate for our basal B-free diet.

Ordinary powdered yeast in bulk was heated for 3 hours in an autoclave at 15 pounds steam pressure. This was then spread out to dry at laboratory temperature and finally reground before using.

POLYNEURITIS.—The symptoms of polyneuritis, as exhibited by our animals, consisted of the characteristic nervousness, followed by loss of appetite, physical instability and finally death. While it was not our general practice to perform autopsies upon B test animals, nevertheless from at least a dozen of the deaths, taken at random, autopsies revealed congestion in the abdomen. Also, in each of these cases the suprarenal glands were found to have atrophied to mere bloody vestiges.

DEPLETION.—At the time when our vitamin-B studies were begun, publications were appearing which discounted the necessity of a depletion period, by reason of the alleged low storage of vitamin B in the animal body. Accordingly, our tests were conducted without depletion of the vitamin-B factor, but in view of the depletion ratio of 1.65 shown by our negative controls, in future work of this kind the depletion of all animals would seem highly advisable.

FEEDING LEVELS.—In previous studies of alfalfa elsewhere daily feeding rations had been used in other laboratories ranging as high as 1.25 grams. For more specific information we used three samples of our alfalfa hays, feeding each of these at three levels, namely 0.25 gram, 0.50 gram and 0.75 gram daily. Ten animals were placed on each level. The hays were good, representative samples of Colorado hay. Later it was found necessary to make preliminary tests of alleged poor-quality alfalfas.

Our preliminary tests disclosed that for vitamin-B assay, a 0.25-gram level of freshly cured alfalfa hay was adequate to produce a slow, steady gain in the weight of young rats. However, a repetition of the tests a few months later, revealed that a 0.25-gram feeding level of the same hays was no longer adequate. Subsequently, a 0.5-gram feeding level for most alfalfas of good quality proved adequate or slightly high. For badly wetted and poorly cured hays, a 0.75-gram feeding level was or was not adequate, depending upon the severity of the curing treatment. It was further found that animals 4 weeks old would not well consume more than 0.50 gram of alfalfa meal daily in addition to the basal diet, and also that animals 8 weeks old would not consume more than 0.75 gram daily. Where levels of 1.0 gram were tried (in case of poor-quality hay) there was much wastage of the food. In a very few instances of exceptionally good alfalfas a 0.25-gram level proved adequate even after several months of storage.

On the basis of our work to date, the indicated optimum level for feeding alfalfa in vitamin-B studies, using non-depleted rats, should range from 0.4 gram to 0.5 gram daily of hays of average good quality. With few exceptions the data in this paper are based on levels of 0.50 gram. In one instance 0.25 gram was adequate; in a few cases it was necessary to use 0.75 gram.

VITAMIN-G TECHNIQUE

The technical studies dealing with the distinction between vitamin-B and vitamin-G factors in foods have progressed so far in recent years that we felt it would be safe to make a study of the G factor in alfalfa hay, while we had to do with the subject of vitamins and our prepared hay samples were available.

G-FREE BASAL DIET 17.—As in case of our other vitamin studies, we experimented with several suggested basal G-free diets, settling finally upon a modification of Sherman and Bourquin's diet as set forth in "The Vitamins" p. 133 (19).

Our modification of this diet consisted in feeding the cod liver oil in smaller quantities and separately from their basal diet. Wherever cod liver oil was used in any of our diets it was always given in daily doses, fresh from the bottle, the obvious reason for this being that in feeding a large number of animals, necessitating a correspondingly large reserve stock of basal diet, the oil, if mixed in the diet would become rancid before the food was consumed.

The G-free basal Diet 17 proved satisfactory in every respect and was of the following composition:

	Percentage
Extracted casein	18
Osborne and Mendel's salts	4
Butterfat	8
"Prepared starch"	70

In addition, 3 drops of cod liver oil were fed daily to animals subsisting on this diet.

The casein used for this diet was prepared in an identical manner as that used for our B-free Diet 4.

Butter of commercial grade was melted and washed with hot water two or three times, and then resolidified and so used.

The starch used in Diet 17 was the same commercial grade as used for our B-free diet, but saturated with an alcoholic extract of wheat kernel (to supply the B factor) after the manner suggested by Sherman and Bourquin (19).

This product comprised our so-called "prepared-starch" component of G-free Diet 17. The diet was usually made up in batches of 2 or 4 kilograms, depending upon the number of animals to be maintained.

SYMPTOMS OF AVITAMINOSIS G.—Altho the symptoms of "avitaminosis G" have been well described by others, we mention our observations here, merely as a matter of record. They consisted generally of a marked loss of hair, in wedge-shaped areas, wide at the caudal and oral extremities and narrowing toward a point at the center of the back. Some animals also showed patches in the flanks and under the hind quarters. There was a marked irritation and scaling off of the skin and in very few instances, skin lesions and sore mouths were observed. No autopsies were performed for internal effects. The animals lost weight very slowly, so that in some instances after a 63-day test period they weighed about 3 grams less than their initial weight at 28 days of age.

DEPLETION.—Our negative controls were fed Diet 17 plus 3 drops of cod liver oil daily and distilled water ad libitum. They were weighed daily up to the point of depletion, which varied in time from 6 to 9 days with an average of about 7.5 days.

FEEDING PROCEDURE.—With the exception of some feeding data on alfalfa seed by McCollum, et al, little was found in the available literature to indicate a desirable feeding level for the biological assay of alfalfa for vitamin-G content. Therefore, using our judgment on the basis of a few preliminary tests, we fed our animals 0.35 gram of alfalfa meal as a daily ration. This was adequate in all cases as results showed. Five animals were fed on each of 31 samples of alfalfa hay, 3 drops of fresh cod liver oil being placed on each daily ration of 0.35 gram. They were weighed daily to the point of depletion and thence twice weekly to the termination of an 8-week period.

In all other respects the daily care of the animals under experiment was the same as that described under vitamin-B procedure. In the work represented in this paper, it was sought to distribute our animals so that not more than one animal per family was fed on a given level of a specific hay. It was also sought as nearly as possible to have an equal distribution of the animals as to sex, altho it was not always possible to carry out these restrictions to the letter.

PART II.—THE DETERIORATION OF ALFALFA HAY

Alfalfa has always been recognized as our most important forage crop and some of the earliest work of this station had to do with the composition of alfalfa hay. Bul. No. 8 by David O'Brine (16), published in 1889, was the earliest of these bulletins. In 1896 Dr. Wm. P. Headden (11) wrote Bul. 35 which gave very complete data on the history and culture of alfalfa and on the chemical composition of the various cuttings. Other bulletins have been published since that time but the analyses quoted are taken either from Bul. 35 or from digestion experiments where the alfalfa fed was analyzed for the particular experiment then being considered.

The question of hay damaged by rain was not considered in Bul. 35 except for one specific case described on pages 11 and 12, quoted in part as follows:

"As this is the only sample of alfalfa hay damaged by rain that we have analyzed, we will make mention of it in this place . . . The total rainfall between May 28 and June 12, the respective dates of cutting and putting into the mow, was 1.76 inches. Any calculations based upon the above without further data would evidently be liable to lead to erroneous conclusions but it suffices to show that the popular estimate of the value of such hay is not far from correct, i.e. about one-half that of good hay. The mechanical losses in such cases are very large . . . The rain fell in three portions; the first fall amounted to 0.31 inch, the second 1.49 inches, and the third 0.26 inch, with intervals of two days or more. The weather was cloudy and warm."

These are the analyses given in Bul. 35:

	Good Hay Percentage	Damaged Hay Percentage
Ash	12.18	12.71
Crude fiber	26.46	38.83
Crude fat	3.94	3.81
Crude protein	18.71	11.01
Nitrogen-free extract	38.71	33.64
	100.00	100.00

This early analysis of damaged hay suggested that an effort be made to determine somewhat more definitely what had been lost and evaluate more carefully the damaged hay. In our attempt to determine the nature of this loss, different phases of the problem were considered.

I. Proximate analyses:
   1. The three cuttings of alfalfa hay.
   2. The early bud, the early bloom and the late-bloom stages of growth.
   3. Hay damaged by 0.5 inch, 1 inch and 2 inches of rain.

II. Determinations of vitamins A, B and G in:
   1. The three cuttings.
   2. The three stages of growth.
   3. The sun-cured hay compared with that cured in diffused light.
   4. The hays damaged by 1 inch and 2 inches of rain.
   5. Hays affected by molds.
   6. Hays grown in other states.

THE SAMPLES OF 1930

Most of the samples of alfalfa hay upon which these vitamin studies were made were grown upon the Experiment Station farm just east of Fort Collins. A plot of common alfalfa, about 16 by 80 feet in area, of excellent stand and having practically no weeds, was staked off and the alfalfa on this plot cut for the early bud, early bloom and late-bloom samples of all three crops when they were ready. This was done to eliminate as many small variations in type of alfalfa and soil as possible.

When cut, the green plants, freed from any adhering soil, were taken to the laboratory and weighed. One sample was spread out to dry in diffused light in the laboratory; another sample was dried out of doors in the sunshine, and three samples were partially dried in sunshine and approximately 0.5 inch, 1 inch, and 2 inches of water applied with a ''rain-maker'' sprinkler—a device used for watering lawns which nearly represents natural rain.

The samples of hay were placed on canvases in a circle around the sprinkler. Five rain gauges were placed between the samples of hay to measure the amount of water applied. It took a little over 1 hour to apply 0.5 inch of water in this manner.

In spreading the hay on the canvas, it was not cocked but spread out in imitation of the swath, tho probably thicker than an ordinary swath, and represented field conditions as nearly as possible.

The samples were then dried in the sunshine, weighed, cut into short lengths, and smaller samples ground quite fine for feeding the rats. This fine grinding is very necessary in order to weigh out small amounts of hay accurately and to prevent the rats separating the leaves from the stems as we wished to determine the vitamins in the whole plants.

Besides the above samples, we obtained two samples from alfalfa fields near Fort Collins which had been damaged by a natural rain. During August, 1930, there occurred a very unusual rainy period of a week when nearly 4 inches of water fell. We found two samples of second cutting hay, one of which had been cocked, and one cut but not raked just before this heavy rain. While there are no basal weights on these samples, they represent field conditions with heavy natural rains doing the damage.

The samples which had been treated with 0.5 inch, 1 inch and 2 inches of water were dried out immediately in the hot sunshine before fermentation could take place. The two field samples (Nos. 3211 and 3212), just mentioned as more nearly representing field conditions, encountered cool cloudy weather with intermittent showers. Under such conditions the hay almost always ferments, allowing enzymes to function and molds to grow profusely.

Samples of 1931

For the 1931 samples we attempted to imitate the moldy field samples and while they were not so moldy as we could wish because they dried out again too quickly, we did obtain some moldy hay, tho we did not pick out this portion but used the entire sample.

We placed some fresh green alfalfa in a beaker under a bell jar and bubbled air thru water to keep the atmosphere saturated with moisture and to remove the carbon dioxide formed by the mold growing on the hay. This hay became very moldy and after a week of this treatment the sample was dried and the vitamin A determined.

Since sunshine seems to have a marked effect on vitamin A, we obtained some samples of hay from Eastern and Southern States to compare with our own. We selected these states since they are in a more humid and dense atmosphere and therefore their hays might show some appreciable differences in vitamin content.

Samples 3234 and 3235 from an Eastern State were the first and second cutting from the same field. They were not as green as our samples and contained more grass and weeds.

Of the three samples from Southern State A, the greenest in color and freest from weeds came from one of the experiment station farms (No. 3238). This was cut during early bloom and was the third cutting. Another sample from state A, 3236, also cut during early bloom, was from the second cutting. The third sample from state A, 3237, was cut during full bloom and was the third cutting. These last two samples were very dull in color and very weedy. All the above out-of-state samples were fertilized.

We obtained two samples, 3241 and 3242, from Southern State B, which were from the same field, one dehydrated and the other cured in their usual way. These were excellent samples of alfalfa hay.

The following table is a list of the samples used and a short description of them.

SAMPLES FROM EXPERIMENT STATION FARM, FORT COLLINS

Lab. No.	Stage of Growth	Cutting	Amount of Rain	Mode of Curing
SEASON OF 1930				
3192	Early Bud	First		Diffused light
3193	Early Bloom	First		Diffused light
3194	Early Bloom	First		Sunshine
3196	Early Bloom	First	1 inch	Sunshine
3199	Early Bloom	First	2 inches	Sunshine
3201	Early Bloom	Second		Sunshine
3202	Early Bloom	Second		Diffused light
3203	Early Bud	Second		Diffused light
3205	Early Bloom	Second	1 inch	Sunshine
3208	Early Bloom	Second	2 inches	Sunshine
3209	Late Bloom	Second		Diffused light
3210	Late Bloom	Second		Sunshine
3213	Early Bud	Third		Diffused light
3214	Early Bud	Third		Sunshine
3216	Early Bud	Third	1 inch	Sunshine
3218	Early Bud	Third	2 inches	Sunshine
3220	Early Bloom	Third		Diffused light
3221	Early Bloom	Third		Sunshine
3223	Early Bloom	Third	1 inch	Sunshine
3225	Early Bloom	Third	2 inches	Sunshine
3227	Early Bloom	Third	2 inches (in cock)	Sunshine
SEASON OF 1931				
3229	Early Bloom	Third		Diffused light (in bell jar)
3230	Early Bloom	Third		Diffused light
3231	Early Bloom	Third	1.5 inches	Sunshine
3232	Early Bloom	Third	1 in. (cock)	Sunshine
3233	Early Bloom	Third	2 in. (cock)	Sunshine
SAMPLES FROM NEAR FORT COLLINS, 1930				
3211	Early Bloom	Second	4 in. (cock)	Sunshine
3212	Early Bloom	Second	4 inches	Sunshine
SAMPLES FROM OTHER STATES, 1931				
			Location	
3234	Early Bloom	First	East	Sunshine
3235	Early Bloom	Second	East	Sunshine
3236	Early Bloom	Second	South A	Sunshine
3237	Full Bloom	Third	South A	Sunshine
3238	Early Bloom	Third	South A	Sunshine
3241	Early Bloom	Third	South B	Dehydrator
3242	Early Bloom	Third	South B	Sunshine

FODDER ANALYSES

The first table groups the analyses with reference to treatment with rain. The following deductions seem justified: That with the increase of rain treatment, there was a subsequent increase of total moisture in the hay obtained. The ash content was decreased with the increase of rainfall, as might be expected, but a 1-inch rain dissolved a surprisingly small amount of the ash constituents. The percentage of crude fiber increased with the amount of rainfall, but here also the amount of change produced by 1-inch of precipitation was very small. The soluble carbohydrates decreased with the increase of rainfall, but again for a 1-inch rain the loss was relatively small. Some rather unusual results occurred in the increase of the protein content of wetted hays. Whether this was attributable to the insoluble character of the proteins themselves or the formation of more proteins in the process was not apparent. Whatever the cause, this increase of protein for a 1-inch rain fell in line with the vitamin-B content of those same samples of hay. Lastly, the ether extract showed a marked falling off with a 1-inch-rain treatment and almost no further loss with 2 inches of rain.

For convenience in comparison, the composition of the alfalfa hays is given under the same headings as are used in the vitamin determinations.

Table 2 gives the effect of maturity of the plant on its composition, the general effects being: 1.—With the progressing age of the plants, there was a gradual diminution in the content of moisture, ash, protein and ether extract. 2.—The soluble carbohydrates seemed to increase slightly up to the early bloom stage and then diminish toward full maturity. 3.—On the other hand, the crude fiber content in all cases increased quite rapidly with the age of the plant.

Table 1.—Comparative Percentage Composition of Alfalfa Hays, Crop of 1930—Effect of Rain. Sun cured.

Sample No.	Stage of Growth	Cutting	Moisture Air-dry	Moisture @100°C	Ash	Crude Fiber	Nitrogen-free Extract	Crude Protein	Ether Extract
3194	Early bloom	First	70.74	5.29	8.18	28.89	37.82	17.53	2.35
3201	Early bloom	Second	75.55	4.87	7.34	34.47	34.02	17.36	1.94
3221	Early bloom	Third	73.37	5.27	8.37	33.68	34.02	17.01	1.65
3214	Early bud	Third	79.07	5.63	9.98	27.27	31.81	23.05	2.26
	Average		74.68	5.26	8.46	31.08	34.41	18.73	2.05
3210	Late bloom	Second	72.12	5.67	7.03	38.43	32.58	14.65	1.64
	(1-inch rain)								
3196	Early bloom	First	68.51	5.35	8.56	30.62	35.83	18.06	1.82
3205	Early bloom	Second	76.43	4.80	7.63	34.01	33.64	17.88	2.04
3223	Early bloom	Third	72.93	6.21	7.11	33.08	34.00	18.23	1.37
3216	Early bud	Third	79.36	5.73	9.55	28.26	32.04	22.87	1.55
	Average		74.30	5.52	8.21	31.49	33.88	19.26	1.69
	(2-inch rain)								
3199	Early bloom	First	70.08	5.38	6.69	32.31	35.95	17.80	1.87
3208	Early bloom	Second	75.75	4.90	8.06	35.46	31.91	17.80	1.87
3225	Early bloom	Third	77.44	6.50	6.35	40.82	29.28	15.78	1.27
3218	Early bud	Third	79.46	6.22	8.54	28.81	31.07	23.75	1.61
	Average		75.68	5.75	7.41	34.35	32.05	18.78	1.65
3227	Early bloom	(cocked) Third	80.02	6.23	10.14	35.65	29.05	17.53	1.40
	(4-inch rain)								
3211		(In Cock) Second		5.92	8.94	45.35	23.57	14.56	1.66
3212		(In Swath) Second		5.90	8.07	40.08	28.15	16.13	1.67

Table 2.—Comparative Percentage Composition of Alfalfa Hays, Crop of 1930—Effect of Maturity.

Sample No.	Stage of Growth	Cutting	Kind Light	Moisture Air-dry	Moisture @100°C	Ash	Crude Fiber	Nitrogen-free Extract	Crude Protein	Ether Extract
3192	Early bud	First	Diff.	76.43	5.35	10.24	26.00	36.40	20.33	1.87
3203	Early bud	Second	Diff.	80.57	5.17	9.43	26.10	31.73	25.41	2.16
3213	Early bud	Third	Diff.	78.35	6.23	9.69	28.29	30.91	22.52	2.36
	Average			78.45	5.58	9.78	26.79	33.01	22.75	2.13
3193	Early bloom	First	Diff.	72.03	5.35	9.52	29.18	36.55	18.22	1.99
3202	Early bloom	Second	Diff.	75.06	4.93	7.54	35.43	32.12	18.06	1.92
3220	Early bloom	Third	Diff.	75.29	5.46	8.43	32.84	34.21	17.45	1.61
	Average			74.12	5.24	8.49	32.48	34.29	17.91	1.84
3200	Late bloom	Second	Sun	69.68	5.25	8.29	31.97	35.97	16.66	1.86
3209	Late bloom	Second	Diff.	72.51	6.17	7.23	40.36	29.88	14.92	1.54

The fodder analyses of the two series of homologous samples (Table 3) did not show any large differences in the ingredients determined. The samples dried indoors contained a slightly higher amount of moisture as might well be expected, where the drying action is not so vigorous. The sun-dried samples contained slightly less of crude fiber and more of soluble carbohydrates than those dried in diffused light. The only other difference of any significance was observed in the protein content. This was slightly higher in the samples dried in diffused light. As stated previously, all of these differences in chemical composition are too small to be considered a real index to the feeding value of specific alfalfa hays. The better index must be sought in the biological assays.

Table 3.—Comparative Percentage Composition of Alfalfa Hays, Crop of 1930—Sun-cured Hay Compared with Hay Cured in Diffused Light.

Sample No.	Stage of Growth	Cutting	Moisture Air-dry	Moisture @100°C	Ash	Crude Fiber	Nitrogen-free Extract	Crude Protein	Ether Extract
		Cured in Diffused Light							
3213	Early bud	Third	78.35	6.23	9.69	28.29	30.91	22.52	2.36
3193	Early bloom	First	72.03	5.35	9.52	29.18	36.55	18.22	1.99
3202	Early bloom	Second	75.06	4.93	7.54	35.43	32.12	18.06	1.92
3220	Early bloom	Third	75.29	5.46	8.43	32.84	34.21	17.45	1.61
3209	Late bloom	Second	72.51	6.17	7.23	40.36	29.88	14.82	1.54
	Average		74.65	5.63	8.48	33.22	32.73	18.21	1.88
			Sun Cured						
3214	Early bud	Third	79.07	5.63	9.98	27.27	31.81	23.05	2.26
3194	Early bloom	First	70.74	5.29	8.18	28.89	37.82	17.53	2.35
3201	Early bloom	Second	75.55	4.87	7.34	34.47	34.02	17.36	1.94
3221	Early bloom	Third	73.37	5.27	8.37	33.68	34.02	17.01	1.65
3210	Late bloom	Second	72.12	5.67	7.03	38.43	32.58	14.65	1.64
	Average		74.17	5.34	8.18	32.55	34.05	17.92	1.97

The tabulated fodder analyses (Table 4) were not very relevant to the question. There did appear to be a diminution of soluble carbohydrates with the progress of the season and especially was this true of the second crop. This crop in Northern Colorado is grown during the heat of the midsummer season. Our summers are dry and there is a high transpiration of moisture at this time. Hence, the second crop would naturally be more woody than either the first or third cuttings. The protein and ether-extract values in all three crops maintained a consistently high level, leaving no basis for adverse deductions.

Table 4.—Comparative Percentage Composition of Alfalfa Hays, Crop of 1930—Seasonal Progression (Stages of Growth).

Sample No.	Stage of Growth	Cutting	Kind of Light	Moisture Air-dry	Moisture @100°C	Ash	Crude Fiber	Nitrogen-free Extract	Crude Protein	Ether Extract
3192	Early bud	First	Diff.	76.43	5.36	10.24	26.00	36.20	20.33	1.87
3193	Early bloom	First	Diff.	72.03	5.35	9.52	29.18	35.74	18.22	1.99
3200	Late bloom	First	Sun	69.68	5.25	8.29	31.97	35.97	16.66	1.86
3203	Early bud	Second	Diff.	80.57	5.17	9.43	26.10	31.73	25.41	2.16
3202	Early bloom	Second	Diff.	75.06	4.93	7.54	35.43	32.12	18.06	1.92
3209	Late bloom	Second	Diff.	72.51	6.17	7.23	40.36	29.88	14.82	1.54
3213	Early bud	Third	Diff.	78.35	6.23	9.69	28.29	30.91	22.52	2.36
3220	Early bloom	Third	Diff.	75.29	5.46	8.43	32.84	34.21	17.45	1.61

All that is apparent from the tabulation of the fodder analyses (Table 5) is that the crude-fiber content is higher than normal, and the carbohydrates are low. The proteins also are somewhat low. Sample 3230 was one of our high-quality hays and is used in this table merely for purposes of comparison. The last three samples, Nos. 3233-27-11, were in appearance very moldy, the poorest hay of all our samples being No. 3211. The analytical results were not very consistently in accord with the degree of moldiness.

Table 5.—Comparative Percentage Composition of Alfalfa Hays, Early Bloom, Crops of 1930-1931—Effect of Molds, Sun-cured.

Sample No.		Inches Rain	Cutting	Moisture Air-dry	Moisture @100°C	Ash	Crude Fiber	Nitrogen-free Extract	Crude Protein	Ether Extract
3230	(1931) Swath	None	Third	76.43	5.71	9.50	29.46	36.14	17.50	1.67
3231	(1931) Swath	1.5	Third	79.39	6.34	10.16	34.52	31.69	16.01	1.25
3212	(1930) Swath	4.0	Second	....	5.90	8.07	40.08	28.15	16.13	1.67
3232	(1931) Cock	1.0	Third	78.71	6.19	10.82	31.82	32.94	16.71	1.50
3233	(1931) Cock	2.0	Third	80.87	5.96	10.19	34.23	31.45	16.53	1.60
3227	(1930) Cock	2.0	Third	80.02	6.23	10.14	35.65	29.05	17.53	1.40
3211	(1930) Cock	4.0	Second	....	5.92	8.94	45.35	23.57	14.56	1.66

If any conclusions may be drawn from the chemical analyses of hays from other states given in Table 6, they are:

(a) The mineral constituents are almost uniformly lower than those of Colorado alfalfa.

(b) The crude fibers in most instances are favorably lower and the carbohydrates favorably higher in the foreign samples.

(c) The protein in some of the foreign samples is considerably lower and in others higher than in Colorado hay. The ash constituents, perhaps, may be considered as the most relevant chemical difference shown.

Table 6.—Comparative Percentage Composition of Alfalfa Hays from Other States, Crop of 1931.

Sample No.	Stage of Growth	Place Grown	Cut-ting	Light	Mois-ture @ 100°C	Ash	Crude Fiber	Nitro-gen-free Extract	Crude Pro-tein	Ether Extract
3230	Early bloom....	Ft. Collins	Third	Diff.	5.71	9.50	29.46	36.14	17.50	1.67
3234	Early bloom....	Eastern	First	Sun	5.32	7.00*	35.06	38.40	12.68	1.51
3235	Early bloom....	Eastern	Second	Sun	5.35	6.53	29.11	44.08	13.21	1.70
3236	Early bloom....	Southern A	Second	Sun	5.17	6.18	32.43	39.95	14.61	1.64
3237	Full bloom.......	Southern A	Third	Sun	5.40	8.10	34.45	37.39	13.03	1.60
3238	Early bloom....	Southern A	Third	Sun	5.33	7.05	26.34	40.81	17.85	2.59
3241	Early bloom....	Southern B	(dehy	drated)	5.72	8.46	24.09	40.59	18.98	2.13
3242	Early bloom....	Southern B	....	Sun	5.44	11.00	25.28	37.84	18.98	1.44

*Contained chromium.

VITAMIN A IN THE HAY SAMPLES OF 1930

Within the past 2 decades the alfalfa plant *(Medicago sativa)* has been examined for its vitamin-A content by a number of workers [1-4, 9, 10, 12, 15(a), 17(c), 18, 19, 21-25, 26 (a, c, d,)]. The results reported thus far indicate an abundance of this vitamin in both the green plant and in the hay. In the process of haymaking it may be reduced to some extent, but good, normal alfalfa hays should contain amounts of vitamin A entirely adequate for livestock. In a study of the effect of rain or weathering on alfalfa hay the importance of vitamin A calls for a consideration of this dietary factor. We have therefore endeavored to obtain quantitative data on the vitamin-A content of our several hay samples, using the rat-growth method.

UNIT OF VITAMIN A DEFINED.—The quantity of vitamins in foods and feeding-stuffs is often expressed in units. Sherman and Smith (19, p. 265) define a unit of vitamin A as "that amount which, when fed daily to standard test animals will suffice to support an average gain in their body weight of 3 grams per week during the test period of 4 to 8 weeks." In making comparisons, it is convenient to express the amount of vitamin A in units per gram of hay. We have done this in the discussion of our results. Units per gram may be easily calculated from the units indicated by the average weekly gains in weight.

EXPERIMENTAL PROCEDURE.—In Part I of this bulletin we present a discussion and description of the technique used in raising, handling and feeding the standard test rats. As may be noted in Part I, a standard rat which had been depleted of its body store of vitamin A was ready to be placed on the experiment. It had its ration of finely ground hay weighed into a small glass "caster cup" containing 0.5 gram dried yeast. The basal diet and distilled water were supplied ad lib., the hay and yeast being fed daily, except Sunday.

THE LEVELS USED.—In placing the depleted rats on the experiment the attempt was made to distribute them so that no two rats of the same litter were placed on the same level of any one hay.

Since individual variations in the experimental animals may be offset to a considerable degree by using sufficient numbers, we tried to utilize the largest feasible group of rats for each sample or level of hay. The proper amount of hay to be fed of any one sample was estimated by first placing five rats on a level which was judged to be adequate. If this proved to be too high, a lower level was tried. Likewise, the weight records resulting from the use of an inadequate level indicated that a higher one should be employed. When the proper level was found, five more rats were placed on it. Our aim was to carry at least ten rats on this optimum (1-unit) level thru the 8-week experimental period and have them show an average net gain corresponding to between 1 and 2 units of vitamin A.

The levels of hay fed daily usually ranged from 0.025 gram to 0.2 gram, intermediate levels of 0.05 and 0.10 gram being most commonly used. In some cases it was found that 0.05 gram was too low, while 0.1 gram was too high. We did not usually attempt to feed at a level between these two.

Data from 625 rats were used in obtaining the figures reported on in this bulletin for vitamin A in the hay samples. Where a certain number of rats was used on a given level of hay, as shown in the tables below, the data afforded by this number of rats were averaged and the resultant figures incorporated into the tables for comparison and discussion. We have included the data of certain rats which died before the end of the experimental period, or which showed a net loss in weight, averaging in their data rather than rejecting them completely. In some cases the statistical study made it preferable to omit the data on certain animals in order to give a more nearly correct probable error.

The average number of rats used per sample, on all levels, exclusive of controls, was 17 plus; an average of 10 rats per sample, on the lowest adequate levels, have yielded the data on vitamin A, detailed below.

THE CONTROLS.—In work of this kind it is necessary to use controls in order to test the worth of the basal diet. The records of six positive controls, covering a period of about 10 months, may be discussed here. These rats received daily, besides the basal diet and yeast, 4 drops of cod liver oil (medicinal grade), the oil being dropped directly on the yeast. These rats all lived the full 8 weeks following depletion and were in excellent condition at the end of that time. Their averages were: Depletion ratio, 2.3; weekly gain, 10 grams; weekly food consumption, 84 grams. Their average growth record is given in Table 9. While their average weight at depletion was only slightly above the average for all groups of rats on adequate levels of hay, their average final weight, 183.6 grams, was the highest of any group. These rats had vitamins A and D supplied in the cod liver oil, and vitamins B and G in the yeast. After considering their records, it seems improbable that these controls suffered from the lack of any food constituent, nitrogenous or otherwise, necessary for normal growth in rats of their age.

Our negative controls, 24 in number, spread over a period of nearly a year and paralleling the experimental animals used for determining vitamin A in our hay samples of 1930, afford evidence that our Diet 14 is free of vitamin A or contains so little that the growing rat cannot survive on that diet alone. The negative controls were continued on the basal diet from the point of depletion until death. Their averages were: Depletion ratio, 2.0; maximum weight, 96.8 grams (only 4.9 grams above their initial weight, 91.9 grams); loss in weight, 8 grams per week; weekly food consumption, 42 grams. They all died, their average length of life after depletion being 18 days; and at autopsy they all showed very definite symptoms of vitamin-A deficiency, altho an autopsy was seldom necessary, the external symptoms being so unmistakable.

Our controls thus established the value of Diet 14, the positives growing normally when supplied with an adequate amount of vitamin A, and the negatives dying with characteristic symptoms of A deficiency when that vitamin was withheld. We may assume, then, that similar experimental standard rats, carried to depletion on Diet 14, and then fed known amounts of alfalfa hay, could survive and gain in weight only if the hay contained vitamin A, since the hay was the only possible source of this vitamin.

THE RESULTS FOR 1930

The results obtained in this work on vitamin A in our several hay samples of the season of 1930 are given below, summarized principally in the form of tables.

Our data depend, we believe, upon the proper application of several of the recognized factors necessary for reliable results, such as reproducibility, variability and probable-error limits, and adequate control of technique. These are all involved when working with living things, and we have endeavored to give them due consideration in our vitamin studies.

In Table 7 are given typical examples of data from the records of individual rats, from which the corresponding data relating to vitamin A have been calculated. The levels, or amounts of the hays fed, were the lowest that seemed adequate for the production of average gains corresponding to 1 to 1.5 units of vitamin A. The table includes also some examples of data from the records of the controls.

Table 7.—Typical Examples of Data on Individual Rats, Determination of Vitamin A on Lowest Adequate Levels, From End of Depletion to End of Experimental Period (all weights in grams).

Rat No.	Sample No.	Level Fed	Initial Weight	Final Weight	Total Loss or Gain	Weekly Loss or Gain	Units A	Life, Days	Ave. Weekly Food Consumption
779	3220	.025	102.0	133.0	+31.0	+4.0	1.3	56	61
974	3213	.05	116.5	145.0	+28.5	+3.6	1.2	56	74
1330	3218	.10	103.0	151.5	+28.5	+3.6	1.2	56	79
997	3212	.20	102.5	136.5	+34.0	+4.3	1.4	56	59
1188	Positive Control	....	94.0	165.5	+71.5	+9.0	....	56	77
449	Negative Control	....	97.0	66.5	−30.5	−15.0	....	16	57

Table 8.—Typical Examples of Semi-Weekly Weight Records of Individual Rats on Lowest Adequate Levels, End of Depletion to End of Experimental Period (on same animals and hays given in Table 7).

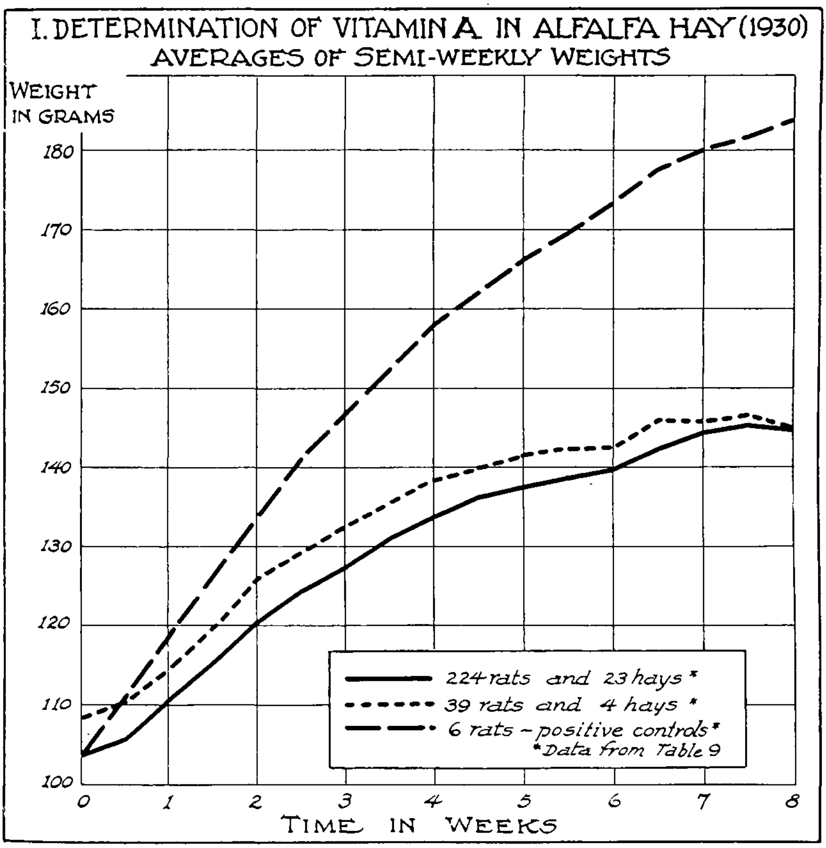
Rat No.	Sample No.	Level Fed																	
779	3220	.025	101.5	102.0	105.0	105.5	110.5	117.5	123.5	126.5	130.5	129.0	133.0	133.5	130.0	132.0	131.5	131.0	133.0
974	3213	.05	119.5	116.5	120.0	125.5	135.5	138.0	141.5	146.5	156.0	158.0	160.5	163.0	168.0	162.5	168.5	162.5	145.0
1330	3218	.10	100.0	103.0	103.0	112.0	117.0	120.0	127.5	131.0	131.0	137.5	143.0	141.0	141.5	146.0	147.0	148.0	151.5
997	3212	.20	102.0	102.5	102.0	99.5	101.0	104.5	109.0	112.0	115.5	119.5	120.5	124.0	125.0	127.0	131.0	134.0	136.5
1188	Pos. Control	....	94.0	98.5	105.0	112.5	118.0	121.5	128.5	133.0	140.0	146.0	146.5	150.5	154.0	160.0	159.0	164.0	165.5
449	Neg. Control	....	97.0	97.5	85.5	81.0	77.5	66.5	....	....	....	....	....	....	....	....	....	....	....

Table 9.—Typical Examples of Average of Semi-Weekly Weight Records, on Lowest Adequate Levels (of same hays given in Tables 7 and 8).

Sample No.	No. Rats																		
3220	10	103.8	105.7	109.9	109.8	121.2	123.8	129.4	133.1	137.0	138.4	139.8	142.0	142.2	142.6	145.1	144.1	142.0	....
3213	10	102.8	106.9	110.2	116.9	121.8	126.5	128.8	131.7	132.7	133.6	138.3	134.7	134.6	143.8	141.4	139.1	136.4	....
3218	9	108.0	110.7	116.6	124.6	127.7	132.3	135.5	137.4	142.8	145.7	145.1	146.2	145.9	148.3	148.3	147.4	147.2	....
3212	10	118.5	118.1	121.2	127.0	132.3	134.6	137.3	141.1	140.8	142.2	144.0	147.6	147.9	150.4	148.8	155.1	152.8	....
Ave.	....	108.3	110.4	114.5	119.6	125.8	129.3	132.8	135.8	138.3	140.0	141.8	142.6	142.7	146.3	145.9	146.4	144.6	....
All Hays (23)		103.6	105.9	110.4	115.1	120.4	124.1	127.1	131.1	133.8	136.0	137.8	138.9	139.7	142.6	144.1	145.2	144.8	....
Pos. Controls		103.9	111.4	118.3	126.4	133.7	141.0	146.8	152.7	157.5	161.9	165.9	169.5	173.3	177.4	180.1	181.7	183.6	....

In Table 8 are shown the semi-weekly weight records of the same six rats listed in Table 7. From such weight records the growth curve of a single rat or of a group of rats may be plotted.

Table 9 presents examples of this kind of average-weight records for groups of rats on the same hays listed in Tables 7 and 8. In Table 9 is also given the average of the semi-weekly weight records for all groups of rats on lowest adequate levels of all hay samples used, 23 in number. For comparison, the averages of the semi-weekly weights of the positive controls are included. A composite growth curve based on averages on the four hays given in Table 9 is shown in Chart I, together with the average for 23 hays. The two hay curves are nearly parallel.



In Table 10 is found, in summarized form, averages from the data obtained on the hay samples of 1930 upon which our chief conclusions may be based, and which also seem to call for the most extended discussion. This table, divided into sections, gives the figures on seven samples of hay cured in diffused light; five samples

Table 10.—Averages From Data by Feeding at Lowest Adequate Levels, for Determination of Vitamin A. (1930 Samples).

Sample No.	Stage of Growth	Cutting	No. Rats	Level Fed, g.	Initial Weight	Final Weight	Total Gain	Weekly Gain	Units A		Life, Days	Weekly Food Consumption
									From Gains	Per g. Hay		
I. Hays Cured in Diffused Light—No Rain												
3192	Early Bud	First	10	.05	85.4	130.4	45.0	5.7	1.8	36.0	56	66
3203	Early Bud	Second	8	.05	82.4	101.0	18.6	2.2	0.8	16.0	50	60
3213	Early Bud	Third	10	.05	102.8	128.1	25.3	2.9	1.2	24.0	52	64
3193	Early Bloom	First	10	.025	81.7	116.5	34.8	4.4	1.5	60.0	56	63
3202	Early Bloom	Second	10	.05	79.8	118.3	38.5	4.8	1.6	32.0	56	67
3220	Early Bloom	Third	10	.025	103.8	142.0	38.2	4.8	1.6	64.0	56	66
3209	Late Bloom	Second	9	.10	111.5	129.6	18.1	2.2	0.9	9.0	53	65
Ave.				.05	92.5	123.7	31.2	3.9	1.3	34.4	54	64
II. Hays Cured in Sun—No Rain												
3214	Early Bud	Third	10	.05	104.0	155.8	51.8	6.5	2.1	42.0	56	74
3194	Early Bloom	First	10	.05	103.6	146.0	42.4	5.3	1.8	36.0	55	68
3201	Early Bloom	Second	9	.05	102.8	123.7	20.9	2.6	0.9	18.0	50	63
3221	Early Bloom	Third	10	.10	108.0	175.1	67.1	8.4	2.8	28.0	56	84
3210	Late Bloom	Second	10	.10	110.6	148.2	37.6	3.7	1.6	16.0	55	70
Ave.				.06	105.8	149.8	44.0	5.3	1.9	28.0	54	72
III. Hays Receiving 1 Inch Rain (in Swath)												
3216	Early Bud	Third	10	.05	106.8	145.3	38.5	4.8	1.6	32.0	56	68
3196	Early Bloom	First	10	.05	80.5	97.2	16.7	2.2	0.8	16.0	53	62
3205	Early Bloom	Second	10	.10	108.3	150.3	42.0	5.7	2.0	20.0	53	69
3223	Early Bloom	Third	10	.20	108.3	168.2	59.9	7.5	2.5	12.5	56	77
Ave.				.10	101.0	140.2	39.2	5.0	1.7	20.1	55	69
IV. Hays Receiving 2 Inches Rain (in Swath)												
3218	Early Bud	Third	9	.10	108.0	145.6	37.6	4.7	1.6	16.0	52	70
3199	Early Bloom	First	10	.20	111.2	149.3	38.1	4.8	1.9	9.5	51	70
3208	Early Bloom	Second	10	.10	119.1	143.3	24.2	3.0	1.3	13.0	55	67
3225	Early Bloom	Third	10	.20	119.3	145.6	26.3	3.3	1.4	12.5	52	67
Ave.				.15	114.4	145.9	31.5	4.0	1.6	12.8	52	69
V. Hay Receiving 2 Inches Rain (in Cock)												
3227	Early Bloom	Third	9	.10	109.4	160.1	51.8	6.5	2.2	22.0	56	72
VI. Hays Receiving 4 Inches Rain												
3212	(Swath)	Second	10	.20	118.5	145.6	27.1	3.2	1.3	6.5	53	69
3211	(Cock)	Second	10	.20	117.0	164.9	47.9	6.0	2.0	10.0	56	75

cured in direct sunlight; hay in the swath, receiving 1 and 2 inches of rain, four samples each; a sample receiving 2 inches of rain, in the cock; and two samples receiving 4 inches of rain each, one in the swath and one in the cock.

Perhaps the most significant items in Table 10 are found in the column headed "Units A Per Gram of Hay," with which we should correlate the data given under the headings, "Stage of Growth," "Cutting," and "Level Fed." For general discussion it may be well to compare these data as given in the several sections of the table. It is to be noted that this table, like the preceding ones, gives data obtained on the "lowest adequate levels."

Inspection of the table indicates that the sample richest in vitamin A is No. 3220, third cutting, at early bloom, cured in diffused light, containing 64 units per gram. The sample lowest in vitamin A is No. 3212, second cutting, in the swath under 4 inches of rain, showing 6.5 units per gram.

Comparing sections I and II of Table 10, we see that, while the samples cured in diffused light average higher in vitamin A than those that were sun-cured, in some single samples the opposite is indicated. The early bloom samples, for all three cuttings, show more vitamin A in the hays cured in diffused light than in those that were sun-cured, whereas the two samples of early bud third cutting, Nos. 3213, and 3214, show less vitamin A in the one cured in diffused light as compared with the other, which was sun-cured. The late-bloom second cutting samples, Nos. 3209 and 3210, seem to show the same thing, but it is possible that those differences are not significant. (See Table 33, section on Statistical Examination). In the early bloom samples, cited above, the difference in favor of curing in diffused light ranges from 40 percent in the first cutting to more than 56 percent in the third cutting. (See Table 11, C).

The remaining sections of Table 10 have to do with samples of hay which, with two exceptions, were treated in the swath with different amounts of rainfall (see section on "The Samples," p. 15).

The samples which received 4 inches of rain, Nos. 3212 and 3211, were, as already noted, from separate fields and from localities other than the Experiment Station farm where our other samples were taken. Hay 3212, in the swath, containing 6.5 units per gram, has been referred to as the lowest of all the samples in the table. Hay 3211 was in the cock, and had become quite moldy. It contained 10 units per gram, not a significant difference statistically, but the living animals and their records indicated that 3212 was not the equal of 3211.

Table 10 shows the relative average values of the hays. The average of all the samples cured in diffused light is seen to be 34.4 units per gram. The next lower value, 28.0 units, is the average for the swath samples (sun-cured). Then follows 20.1 units for 1 inch of rain, and 12.8 units for 2 inches, both in the swath. Curing alfalfa in the sun, without rain, seems to have almost as much of an adverse effect on the vitamin-A content as 1 inch of rain.

The average levels fed, as given in Table 10, are of interest. The lowest figure is 0.05, the average for all levels of hay cured in diffused light; the other averages follow in order: 0.06 for sun-cured, 0.1 for 1 inch of rain, and 0.15 for 2 inches of rain.

Since the effect of rain upon the hay is of paramount importance in this investigation, the data in Table 10 bearing on this point have been condensed and reassembled in Table 11, A, in order that this effect may be more readily apparent. As the sun-cured samples more nearly represent field conditions than the samples cured indoors out of direct sunlight, the former have been used as the basis from which the percentage losses from rain have been calculated. The four sets of samples seem to indicate, in general, progressively lower amounts of vitamin A with greater amounts of rain. The percentage of loss of vitamins from the hays under rain appears to be significant in the cases of the first two sets, altho the differences are probably not so significant in the case of the third set.

Table 11.—Comparative Differences in Vitamin-A Content of Alfalfa Hay.
A.—Effect of Rain

Sample No.	Stage of Growth	Cutting	Lowest Adequate Level	Treatment	Units per Gram of Hay	Difference in Percentage of Vitamin A
3214	Early Bud	Third	.025	Sun-cured	42.0	
3216	Early Bud	Third	.05	1 in. Rain	32.0	−23.8
3218	Early Bud	Third	.10	2 in. Rain	16.0	−61.9
3194	Early Bloom	First	.05	Sun-cured	36.0	
3196	Early Bloom	First	.05	1 in. Rain	16.0	−55.6
3199	Early Bloom	First	.20	2 in. Rain	9.5	−73.6
3201	Early Bloom	Second	.05	Sun-cured	18.0	
3205	Early Bloom	Second	.10	1 in. Rain	20.0	+11.1
3208	Early Bloom	Second	.10	2 in. Rain	13.0	−27.8
3221	Early Bloom	Third	.10	Sun-cured	28.0	
3223	Early Bloom	Third	.20	1 in. Rain	12.5	−55.4
3225	Early Bloom	Third	.20	2 in. Rain	12.5	−55.4
3227	Early Bloom	Third	.10	2 in. Rain (Cock)	22.0	−21.4

B.—At Early Bud and Early Bloom Stages of Growth—No Rain

Sample No.	Stage of Growth	Cutting	Lowest Adequate Level	Treatment	Units per Gram of Hay	Difference in Percentage of Vitamin A
3192	Early Bud	First	.05	Diff. Light	36.0	
3193	Early Bloom	First	.025	Diff. Light	60.0	+66.6
3203	Early Bud	Second	.05	Diff. Light	16.0	
3202	Early Bloom	Second	.05	Diff. Light	32.0	+100.0
3213	Early Bud	Third	.05	Diff. Light	24.0	
3220	Early Bloom	Third	.025	Diff. Light	64.0	+166.6

C.—Cured in Diffused Light and in Direct Sunlight—No Rain

Sample No.	Stage of Growth	Cutting	Lowest Adequate Level	Treatment	Units per Gram of Hay	Difference in Percentage of Vitamin A
3193	Early Bloom	First	.025	Diff. Light	60.0	
3194	Early Bloom	First	.05	Sun-cured	36.0	−40.0
3202	Early Bloom	Second	.05	Diff. Light	32.0	
3201	Early Bloom	Second	.05	Sun-cured	18.0	−43.7
3220	Early Bloom	Third	.025	Diff. Light	64.0	
3221	Early Bloom	Third	.10	Sun-cured	28.0	−56.2
3209	Late Bloom	Second	.10	Diff. Light	9.0	
3210	Late Bloom	Second	.10	Sun-cured	16.0	+43.7
3213	Early Bud	Third	.05	Diff. Light	24.0	
3214	Early Bud	Third	.05	Sun-cured	42.0	+42.9

In the fourth set, Table 11, A, Nos. 3227 and 3221 show nearly the same vitamin-A content, altho the latter was sun-cured and the former had 2 inches of rain, in the cock. No. 3227 should also be compared with No. 3225, which received 2 inches of rain, in the swath. The former, on the 0.1-gram level, shows 22.0 units of vitamin A per gram, while the latter, on the 0.2-gram level, shows 12.5 units, a difference of more than 43 percent.

The superiority of 3227 over 3223 and 3225 was easily seen while feeding and weighing the rats on those hays, before the experimental period or any calculations had been completed. No. 3227 was off-color, moldy, with a bad odor, and was apparently of low value, yet the rats ate it readily and thrived on it.

Table 11, A, indicates that among the early bloom sun-cured samples, the first cutting carries the most vitamin A, and that it suffers the greatest loss of that vitamin under rain. The third cutting appears to stand next, and the second cutting last, in this respect. In contrasting the two stages of growth the early bud third cutting ranks first, with 42.0 units, but under 2 inches of rain this hay lost heavily in vitamin A.

In Table 11, B, we may see how much higher, in percentage, the early bloom samples run in vitamin A as compared with the early bud samples, all cured in diffused light.

In Table 11, C, we may note the differences in percentages of vitamin A in the sun-cured samples as compared with those cured in diffused light. In the early bloom samples these differences appear to be quite significant. The early bloom stage, third cutting, carries more vitamin A than either the early bud or the late-bloom stage; and for all three cuttings the early bloom stage, in diffused light, is consistently higher than the sun-cured. The significance of the apparent differences in vitamin-A content of the late-bloom second cutting and the early bud third cutting has been referred to above.

Table 12.—Comparison of Averages of Summarized Data on Vitamin A, Alfalfa Hay Samples of 1930.

	No. Averaged	Initial Weight	Final Weight	Total Loss or Gain	Weekly Loss or Gain	Units A	Life, Days	Weekly Food Consumption
On Lowest Adequate Levels	23 Hays	103.7	145.5	+41.8	+ 5.2	1.7	54	69
Pos. Controls	6 Rats	103.9	183.6	+79.7	+10.0		56	84
On Highest Inadequate Levels	20 Hays	100.7	99.6	+ 0.2	− 1.3	0.4	45	53
Neg. Controls	24 Rats	91.9	72.2	−19.7	− 8.0		18	42

The choice of the data used in Tables 10 and 11 was fixed by consideration of data from inadequate levels, not shown in tables, and of data from levels which on trial proved to be too high.

In Table 12 are given averages of the summarized data on the 1930 samples. Direct comparison is made of the figures from feeding the 23 hays on the lowest adequate levels with the positive controls carried at the same time. The 20 hays on the highest inadequate levels are compared with the negative controls.

THE SAMPLES OF 1931

These samples have been described above and we here report upon the results of determining vitamin A in them. The technique of using standard rats, both for the controls and the vitamin assay was the same as with the hays of 1930. Eight positive and 12 negative controls were used which gave records entirely parallel with the controls of 1930.

In Table 13 are shown the data obtained with the Colorado samples, Nos. 3229-3233. Among these the greatest loss of vitamin A was shown by the slightly moldy sample No. 3231, under 1.5 inches of rain, in the swath. This difference is more than 76 percent. The two samples from the cock, and especially the one from the bell jar, were decidedly moldy. The differences in the vitamin-A content of these three were practically insignificant, as the sample under 2 inches of rain, in the cock, carried 38 units, the one under 1 inch of rain had 40 units, and the one from the bell jar, 32 units per gram.

These results are in line with those obtained on the 1930 samples, among which the moldy samples in the cock, under rains, apparently lost less vitamin A than those rained on in the swath. We have reason to believe that No. 3229, the bell-jar sample, when first prepared was rather higher in vitamin A than some months later.

The data on the hays from other states, found in Table 14, Nos. 3234-3242, are largely self-explanatory. It should be mentioned, however, that in the cases of Nos. 3234 and 3237 the levels fed were really inadequate. With the latter sample, from a Southern State, it was found that the relatively high level of 0.2 gram gave data indicating 2.5 units per gram. A level of 0.25 gram was then tried, but this was even less satisfactory.

Tables 13 and 14.—Averages from Data by Feeding at Lowest Adequate Levels, for Determination of Vitamin A. (1931 Samples, Early Bloom Stage).

Sample No.	Locality	Cut-ting	Treatment	No. Rats	Level Fed	Initial Weight	Final Weight	Total Loss or Gain	Weekly Loss or Gain	Units A		Life Days	Weekly Food Con-sumption
										From Gains	Per g. Hay		
3230	Fort Collins	Third	Diff. light	10	.025	105.6	150.8	45.2	5.7	1.9	76.0	56	70
3231	Fort Collins	Third	1.5″ Rain (Swath)	12	.05	110.3	127.2	16.9	2.0	0.9	18.0	54	63
3232	Fort Collins	Third	1″ Rain (Cock)	10	.05	100.1	147.0	46.9	5.9	2.0	40.0	56	71
3233	Fort Collins	Third	2″ Rain (Cock)	10	.05	99.2	143.7	44.5	5.7	1.9	38.0	52	66
3229	Fort Collins	Third	Moldy, from bell jar	14	.025	108.5	119.3	10.6	0.8	0.8	32.0	51	60
	(Table 14)												
3234	Eastern State	First	Sun-cured	15	*.10	106.1	103.4	−27	−1.5	0.5	5.0	43	61
3235	Eastern State	Second	Sun-cured	5	.10	95.1	152.2	57.1	7.2	2.4	24.0	56	79
3236	Sou. State, A	Second	Sun-cured	12	.10	105.2	123.1	18.0	2.5	0.9	9.0	53	62
3237	Sou. State, A	Third**	Sun-cured	11	*.20	109.4	110.8	1.3	−0.9	0.5	2.5	50	54
3238	Sou. State, A	Third	Sun-cured	11	.05	112.8	127.4	14.6	9.6	0.9	18.0	51	64
3241	Sou. State, B		Dehydrated	11	.025	107.2	142.1	35.0	4.6	1.5	60.0	53	70
3242	Sou. State, B		Sun-cured	11	.05	107.5	131.5	24.0	1.7	1.2	24.0	49	63
			Pos. Controls	8		104.3	169.6	65.3	8.2			56	78
			Neg. Controls	12		113.0	84.1	−29.0	−10.0			25	46

* Inadequate.
**Full Bloom.

The first-cutting sample, No. 3234, from an Eastern State was found to contain chromium in the ash. This abnormality is not yet explainable, but it may account in part for the unsatisfactory result obtained in determining the vitamin-A content. A level of 0.1 gram seemed to be high enough at first, but after carrying 15 rats on this level, 12 of them showed that either the level was too low, or the utilization of vitamin A was interfered with.

In the case of the second-cutting sample, No. 3235, from the same state as No. 3234, five rats indicated that the 0.1-gram level was too high, and 10 rats that the 0.05-gram level was too low. Several months later the 0.075-gram level was tried, but this amount was no better. Our experience leads us to suspect that this sample, and possibly others, deteriorated in vitamin content due to the way in which they were stored and handled. Changes in the amount of vitamin A in alfalfa, on storage, are to be investigated further.

The Southern State sample, from the dehydrator, shows 60 units per gram, comparing favorably with some of the best Colorado samples. It is 60 percent higher in vitamin A than the corresponding field-cured sample from the same state (No. 3242), showing 24 units per gram, which in turn is higher than the best sample from the other Southern State (No. 3238), the same as the best sample from the Eastern State (No. 3235), and lower than the Colorado samples receiving rain in the cock.

Table 15.—Comparison of Vitamin-A Content of Alfalfa Hay, Early Bloom Third Cutting, Seasons of 1930 and 1931.

Sample No.	Year	Treatment	Units Per Gram of Hay
3220	1930	Diffused Light	64.0
3230	1931	Diffused Light	76.0
3223	1930	1 inch Rain (Swath)	12.5
3231	1931	1.5 inches Rain (Swath)	18.0
3227	1930	2 inches Rain (Cocked)	22.0
3233	1931	2 inches Rain (Cocked)	38.0

In Table 15 a comparison is made of some of the early bloom third-cutting samples, seasons of 1930 and 1931. In both seasons the highest vitamin-A content was found in this cutting and this stage of growth, when cured in diffused light. The two quantities, 64 and 76 units, are practically and statistically the same. The effects of rains are somewhat comparable; while not equal, they tend toward the same relative result. We do not, of course, assert that there is necessarily any constancy in such results from year to year.

In the tables, pages 65 and 66, the results on the study of the vitamin-A content of alfalfa hay are further summarized and condensed for the purpose of showing the relative amounts in our hay samples for the two seasons.

The results on vitamin A for all samples cured in diffused light, for both 1930 and 1931, without regard to cutting, stage of growth, or level fed, give an average of 39.8 units per gram. The sun-cured samples average 22.0 units; those receiving 1 inch of rain in the swath average 20.1 units, while the samples which had 2 inches of rain in the swath average 12.8 units. These figures show a difference of 27 units per gram, or more than 67 percent, in favor of the hay cured in diffused light as compared with that which had received 2 inches of rain in the swath.

VITAMIN-A VALUES OF ALFALFA HAY

The averages given just above suggest the importance of attempting to control haymaking so as to conserve the vitamin A. The proper practice to follow is indicated to some extent by the fact that deterioration of alfalfa hay, with respect to vitamin A, begins very soon after cutting, if the hay is sun-cured. Further and more serious deterioration occurs if the hay is exposed to heavy rains, or intermittent showers, followed by sunny weather. The damage is clearly less if the hay is in the cock, rather than in the swath.

In judging the vitamin-A value of any given hay it is desirable to know its source, which cutting, its stage of growth when cut, conditions of curing, and any untoward conditions affecting the growing plant, such as hail, frost, or pests. It seems not impossible to place a rational evaluation on a hay when the above details are known. Setting up certain specifications or standards which a hay must meet in order to attain a certain grade or price would tend to emphasize the importance of the vitamin content because we are beginning to learn what conditions and factors favor the conservation of the vitamins, and how the vitamins may be lost with a consequent adverse effect on the quality.

VITAMIN A AND GROWTH

The testimony of a great many workers with vitamin A indicates that when this vitamin is abundantly supplied to a young animal it exerts a definite and notably favorable effect on that animal's growth and well-being. It is consequently regarded as a necessary growth factor, altho it is possible that it may not be a single substance, and may have a variety of functions. The records of our rats seem to show that, as they became older and grew heavier, the demand for vitamin A was greater. Of all groups of rats, our positive controls made the most rapid growth and showed the highest average food consumption, 91 grams of basal diet per week. Their 4 drops of cod liver oil per day no doubt supplied an abundance of vitamin A, stimulating growth, which in turn stimulated the appetite, resulting in a heavy consumption of food.

The rats fed the lowest adequate levels of hay often gave growth curves showing a slackening in rate of growth about the fifth or sixth week. The curve would then take a sudden upward trend, flattening again toward the end of the eighth week. These fluctuations may have had a relation to the amount of vitamin A which the rats were able to store during the early weeks of the experiment.

The experience of other workers having shown that in general it may be unprofitable to continue the experiment more than 8 weeks, we confined our experiments to that length of time. In most cases, the rats that failed to complete the 8-week period showed either a very low average weekly gain, or a loss, chiefly the latter.

In this connection the average weight of basal diet consumed per week should be considered, as this is often a significant figure. The rats when close to the critical or danger limit in the levels of hay fed were, as a rule, subnormal in health and vigor, which includes appetite and digestion. The property of vitamin A, among others, to enable the animal to resist infection is a factor of great importance, for pathogenic bacterial invasions of the body are bound to affect appetite and body weight. When a rat sickens from the lack of vitamin A, permitting infections, it eats and drinks less and less, steadily loses weight, and finally dies. (See also under "Symptoms," and "Autopsies," Part I, p. 10.)

CAUSES OF LOSS OF VITAMIN A FROM ALFALFA

Our work seems to confirm the conclusions arrived at by others (2, 10, 18) that curing in sunlight tends to lower the vitamin-A content of alfalfa. Further, if we think of vitamin A as being fat-soluble, rather than water-soluble, we naturally inquire into the reasons for the loss of this vitamin from hay when exposed to heavy rains. Such factors as ultraviolet rays, chemotropic effects and oxygen or ozone acting on the damp hay may be involved. Bacteria, molds and enzymes may likewise play an important part, or there may be several of these factors acting together. Aeration and conditions of storage probably have effects which, while relatively slow in action, may still quite definitely change the vitamin content of the hay. The work of Shuey (20) in 1914, suggests a possible relation between the diastase and vitamin-A contents of alfalfa hay. This remains to be worked out. The reports by Hauge and Aitkenhead (10) are also suggestive as to the relation of enzymes to losses of vitamin A during the curing of alfalfa, especially in the field. Whatever the causes, the effects of weathering, principally sun, rain and air, result in the deterioration of alfalfa hay with respect to the vitamin-A content.

CONCLUSIONS

Our work thus far on vitamin A in alfalfa hay seems to indicate the following conclusions:

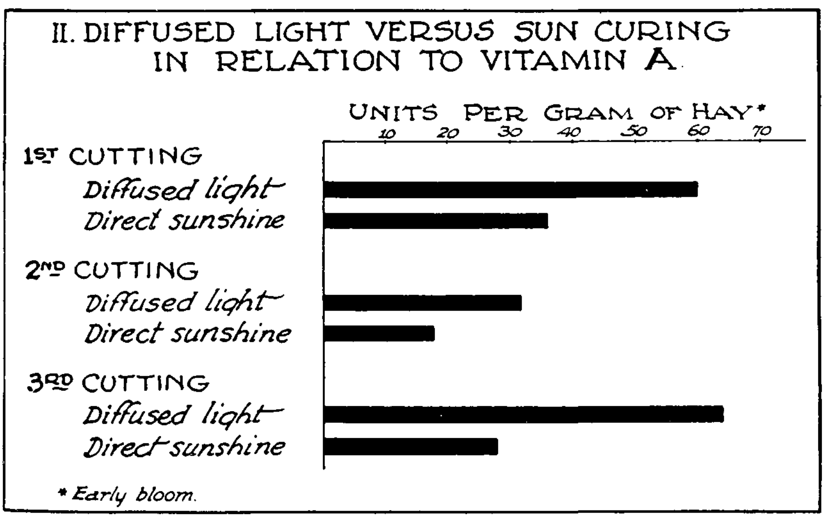
1. Hay cut at the early bloom stage contains more vitamin A, as a rule, than that cut at other stages of growth, the method of curing being the same.

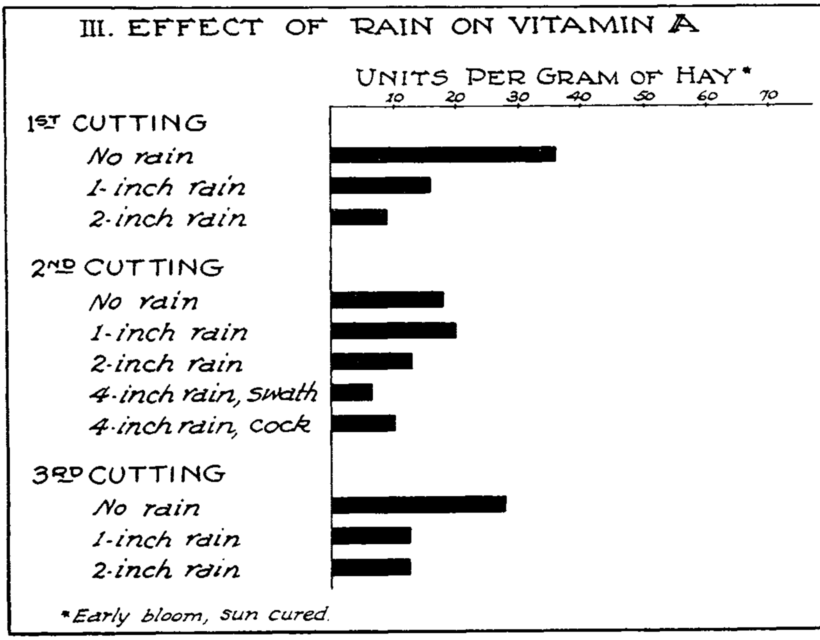
2. Hay cured in diffused light is superior, in most cases, to that cured in direct sunlight.

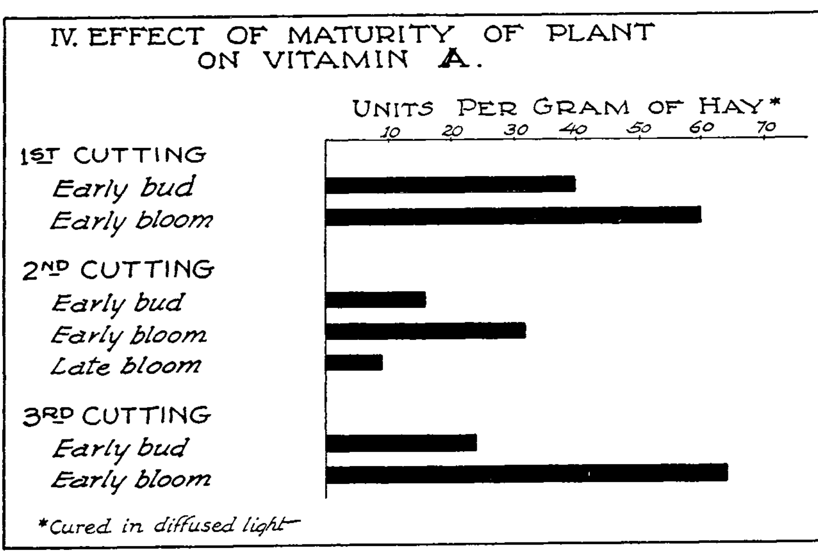
3. The largest amount of vitamin A was found, for 2 successive years, in the third cutting, early bloom stage, cured in diffused light. The first cutting, early bloom stage, in 1930 showed a slightly lower content than the third crop, while the second crop contained half as much.

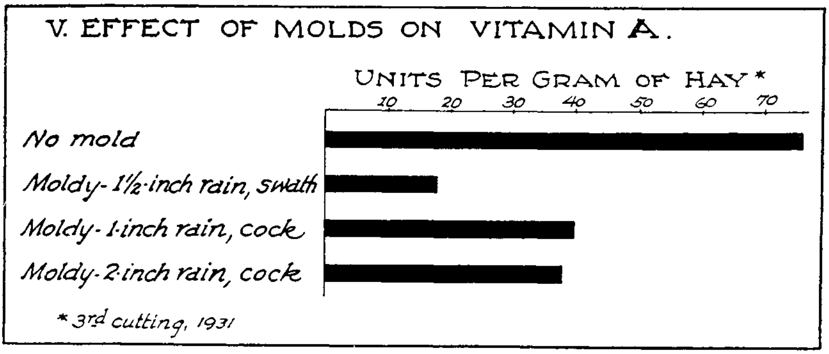
4. While 1 inch or more of rain quite definitely lowers the vitamin-A content, this damage is less if the hay is in the cock instead of in the swath.

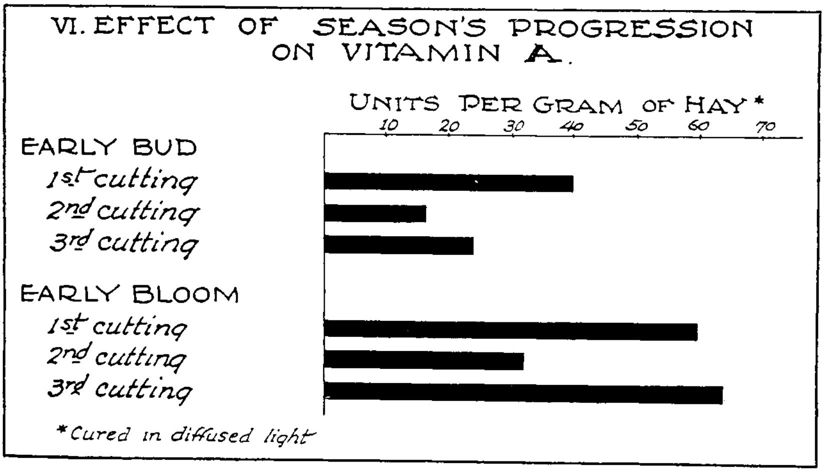
5. Colorado alfalfa showed a higher content of vitamin A than the samples grown in certain other states in 1931.




III. EFFECT OF RAIN ON VITAMIN A
UNITS PER GRAM OF HAY*
10 20 30 40 50 60 70
1ST CUTTING
No rain
1-inch rain
2-inch rain
2ND CUTTING
No rain
1-inch rain
2-inch rain
4-inch rain, swath
4-inch rain, cock
3RD CUTTING
No rain
1-inch rain
2-inch rain
*Early bloom, sun cured.


IV. EFFECT OF MATURITY OF PLANT ON VITAMIN A.
UNITS PER GRAM OF HAY*
10 20 30 40 50 60 70
1ST CUTTING
Early bud
Early bloom
2ND CUTTING
Early bud
Early bloom
Late bloom
3RD CUTTING
Early bud
Early bloom
*Cured in diffused light



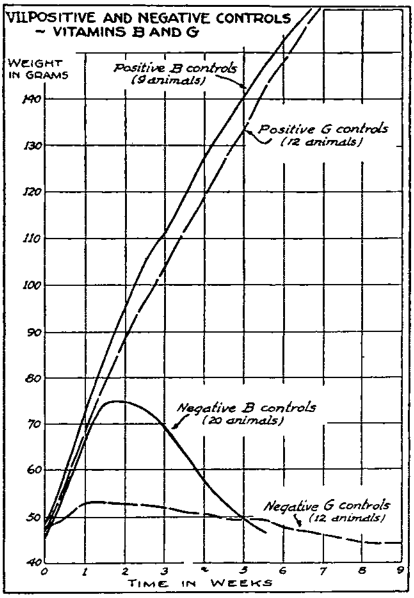


RESULTS OF VITAMIN-B STUDIES

NEGATIVE CONTROLS.—Both albino and black-hooded rats were used in this work. When our vitamin work was first undertaken, we procured stock animals from several sources in order to start a good strong strain of our own.

An aggregate of 20 negative controls, starting with an average initial weight of 45.3 grams, attained a maximum of 74.8 grams at the end of 10 to 14 days, as is shown in Table 16. This was an average gain of 29.5 grams up to the point of depletion. The final average weight of these animals at death was 46.7 grams, or 1.4 grams above their initial weight. After the fourteenth day, they gradually declined and died at between 27 and 35 days. The average life period on our Diet 4 was 32.7 days.

On the basis of the above figures, the depletion ratio of the animals was 1.65, indicating a considerable storage of vitamin B in young rats. Very few of the negative controls survived to the point of their initial weight in the downward trend of their weight curve. They had subsisted solely upon basal Diet 4 and distilled water ad libitum, plus 3 drops of cod liver oil daily.



POSITIVE CONTROLS.—As is shown in Table 16, a total of nine positive controls, started with an average initial weight of 48.6 grams, gained steadily thruout the 8-week experimental period at an average weekly rate of 15.3 grams, attaining a maximum of 171.3 grams in 56 days. Their diet differed from that of the negative controls by 0.5 gram of powdered yeast, given daily. These animals were in excellent condition of health at all times, equal to our young stock rats, which usually weigh from 175 to 200 grams at 12 weeks of age. In fact, several of them were subsequently used as breeding stock to good advantage.

EXPERIMENTAL ANIMALS

In the description of our methods and technique, appended to this paper, it is stated that our experimental animals in vitamin-B work were not depleted before placing them on test. The reason for this, which at that time seemed valid, has since been shown to be unsound. The results obtained in our laboratory by the non-depletion tests enabled us, quite successfully, to align our various samples of alfalfa hay in accordance with the magnitude of their vitamin-B content, but the values as expressed in actual units of vitamin per gram of hay would require a re-check to establish accuracy. (About 1 year after our data for this paper were accumulated, such a re-check was made on four of the original hay samples and the results and further comments on them will be found as Table 30, p. 58.

GENERAL COMMENTS

The literature available to us and dealing with the studies of vitamin-B content in alfalfa was of a too general and purely qualitative nature to be of much assistance in the investigations here presented.

Table 16.—Control Animals—Vitamin B Semi-Weekly Gain Chart (grams)—1930-31.

No. of Animals	Legend																	
20	Negative "B"......	45.3	56.1	66.9	74.1	74.8	72.9	69.1	63.0	57.7	52.6	49.5	46.7	All died.				
9	Positive "B"........	48.6	60.3	73.8	85.9	96.3	105.5	111.3	119.4	128.0	134.2	140.6	147.0	152.4	158.0	163.3	168.3	171.3

Fate of Animals

No. of Animals	Legend	Initial Weight	Maximum Weight	Final Weight	Days on Experiment	Food Consumed	Animals Died	Average Weekly Gain
20	Negative "B"...........	45.3	74.8	46.7	32.7	207.5	20	+.2 g. } 8 weeks exp.
9	Positive "B".............	48.6	171.3	171.3	56.0	643.6	None	15.3 g. } 8 weeks exp.

The burden of most of these investigations was a qualitative comparison of the alfalfa plant with other plants (17,(b) wherein large percentages of the plants or roots in question were admixed with the remainder of the total diet. Little is said of wastage of the food. It appears to be the opinion of several workers that about 15 to 20 percent of alfalfa is necessary in the diet of animals in order to assure normal growth (15(b), (24) as related to vitamin-B content.

Osborne and Mendel (17) made a comparison of the vitamin-B content in the green growing plants of clover, timothy, alfalfa, etc., showing alfalfa to be richer in vitamin B than either clover or timothy. E. B. Hart, et al (8), in some feeding tests with Holstein cows, found that by supplementing an otherwise heavy grain diet with adequate amounts of alfalfa hay, the cows were able to produce at least one calf before reproductive weakness set in. They ascribed the value in the alfalfa largely to the presence of high quantities of mineral salts.

Many attempts have been made by specific or quasi-chemical tests to work out a technique for the quantitative measurement (7) and even the segregation of the vitamin-B factor. It has even been attempted to measure the quantity of B vitamin in alfalfa (6) and other plants by growing yeast cultures in the plant extracts.

Our work as represented in this paper pretends at nothing more ambitious than an attempt to answer certain practical questions with regard to the nutrient value of alfalfa hays. Since simple chemical tests such as fodder analyses do not adequately tell the story and since the technique of biological assay has now been perfected to the degree that in many instances it may be said to be quantitative, it was by this avenue that we sought to evaluate the quantity of vitamin B contained in alfalfa hays, and further, to show how that quantity may be affected by the exigencies of various methods of curing.

EFFECT OF RAIN ON THE CURING OF ALFALFA

The effect of rain upon alfalfa hays in the curing process was very interesting if not in all respects logical, as brought out by our tests. Altho hays dried in diffused light were generally of higher vitamin-B content, yet it was known that if partially cured hay were wetted it would be difficult to dry such hay in diffused light with sufficient celerity to prevent the formation of molds. The formation of molds in itself constitutes a factor in the curing of alfalfa. And so, to avoid complications, the comparisons drawn were based on sun-cured hays.

This series of hays (Table 17) represented four homologues from each of our three crops. One set had no rain at all and was sun cured. A second set had each received 1 inch of rainfall when partly dry and a third set of samples had each received 2 inches of rainfall. All were subsequently dried in the sunlight as quickly as possible to prevent molding.

Besides these, we collected from farms in the community, two samples of second-crop alfalfa which had received 4 inches of natural rainfall. In August of 1930, an unusual thing had happened in the vicinity of Fort Collins. Just after the farmers had cut their second alfalfa crop, beginning on August 9 and continuing intermittently for about 8 days, there came a series of rains, which, taken together, amounted to almost 4 inches of precipitation. One of these samples (No. 3211) had been cocked, ready for stacking, while the other (No. 3212) had been freshly cut when this series of rains overtook them. The two samples were brought in and dried as quickly as possible in the direct sunlight, as all other samples of this series had been.

Table 17.—Biological Assay—Vitamin B—Alfalfa Hays, Crop of 1930—Sun-cured—Rain in Field vs. No Rain.

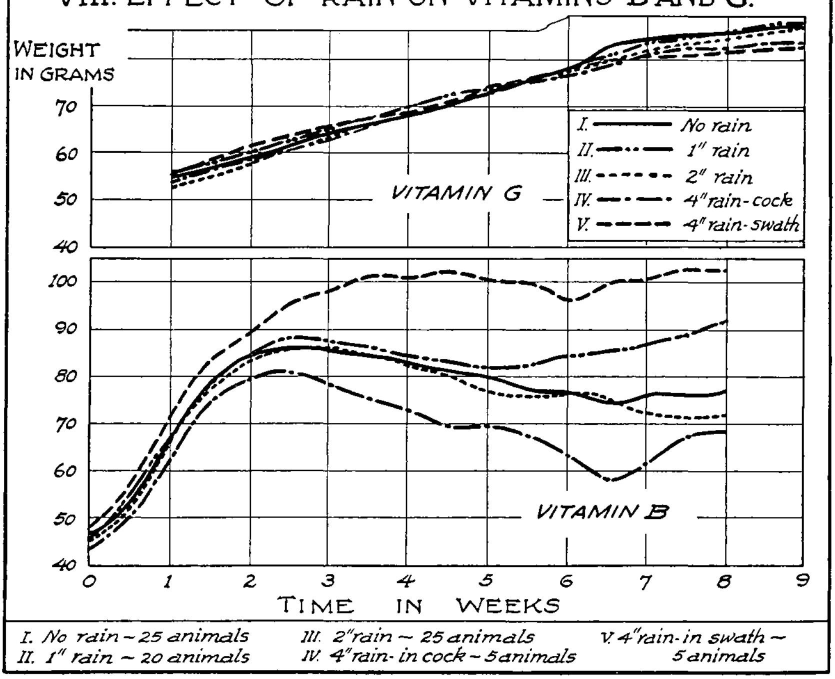
Sample No.	Stage of Growth	Cut-ting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Con-sumed
No Rain										
3194	Early Bloom	First	5	.50 g.	46.0	72.8	3.1	2.0	52.6	325 g.
3201	Early Bloom	Second	5	.50	49.4	80.9	3.9	2.6	55.8	364
3221	Early Bloom	Third	5	.50	43.1	63.6	2.5	1.7	52.0	324
3214	Early Bud	Third	5	.50	50.0	80.0	3.7	2.5	55.6	395
	Average				47.1	74.3	3.3	2.2	54.0	352
3210	Late Bloom	Second	5	.25	47.0	74.6	3.4	4.6	56.0	372
1 Inch Rain										
3196	Early Bloom	First	5	.50	50.6	119.2	8.5	5.7	56.0	455
3205	Early Bloom	Second	7	.50	42.2	80.9	4.8	3.2	56.0	374
3223	Early Bloom	Third	5	.50	44.2	59.7	1.9	1.3	48.2	310
3216	Early Bud	Third	5	.50	48.0	98.8	6.3	4.2	56.0	423
	Average				46.2	89.6	5.4	3.6	54.0	391
2 Inch Rain										
3199	Early Bloom	First	5	.50	45.1	87.5	5.3	3.5	56.0	390
3208	Early Bloom	Second	5	.50	45.3	64.6	5.6	3.7	53.8	365
3225	Early Bloom	Third	5	.75	46.2	80.2	4.2	1.9	54.4	383
3218	Early Bud	Third	5	.50	46.1	65.6	2.4	1.6	55.6	380
	Average				45.7	74.5	4.4	2.7	54.9	379
3227	Early Bl. (Cock)	Third	5	.75	44.2	53.8	1.2	.5	43.8	284
4 Inch Rain										
3211	In Cock	Second	5	.75	43.3	61.2	2.2	1.0	48.6	298
3212	In Swath	Second	5	.75	47.8	102.8	6.8	3.0	56.0	441

Sample No. 3211 was badly molded and malodorous when taken, while No. 3212, aside from having a brown color, appeared to be undamaged. We did not have all of the basic data for these two samples and hence full comparisons were impossible.

While the chemical losses as shown by fodder analyses, Table 1, were consistent, nevertheless they were too small in themselves to account for the discrepancy in food value indicated by the hays. In one respect they seemed to confirm the biological assays, namely, in the nitrogen content.

The units per gram of vitamin B in the three sets of homologues correspond directly with the fodder analyses in that 1 inch of rainfall seemed to improve appreciably the food quality of the hays tested, and further, that even those samples which had received a 2-inch rainfall were of better quality in some instances than the same samples dried in direct sunlight without any rain. The two extraneous samples which had received 4 inches of natural rainfall brought out some interesting facts. Sample No. 3212 had lain in the swath for 8 days before the weather cleared enough to permit sampling. The only apparent damage it had suffered was a partial loss of the sweet odor of fresh hay and had a brown instead of green coloration of the dried leaves. As Table 17 and also the growth curve (Chart VIII) show, this hay contained more units of vitamin B per gram than several other sun-cured hays which were dried under ideal conditions.

VIII. EFFECT OF RAIN ON VITAMINS B AND G.



Sample No. 3211, on the other hand, had been dried and cocked, prime for stacking. After 8 days of showers, this hay was thoroly soggy, dark gray-green in color, moldy, and had an offensive odor of decay. The farmer owner of the hay ventured the information that when dried this hay would be used as roughage in feeding sheep during the succeeding winter.

The vitamin-B content of this sample was roughly about a third of that indicated by sample No. 3212. The contrasts between these two as shown by Table 17 and Chart VIII are striking. How much of this difference could be attributed to the presence of molds and how much to other causes remains a question.

EFFECT OF DEGREE OF PLANT MATURITY ON ALFALFA HAY

Altho our library facilities are not exhaustive, we found but one reference (17(b) to work in which Osborne and Mendel (1919) found in some qualitative tests that hay from immature alfalfa was richer in vitamin B than was hay from more mature plants. When the hays used in our feeding experiments were classified to show the effects of degree of maturity (of plant at time of cutting), some interesting relations between the fodder analyses and the biological assays were brought out. These are shown in Tables 2 and 18.

The hays used in this series of tests represent two stages of growth of each of the three crops usually harvested in Northern Colorado. They were the immature or early bud stage and the second or early bloom stage. The latter represents the age at which it is customary to harvest the crop in this region. All of these samples were cured in diffused light, which process invariably gave us hays of better quality.

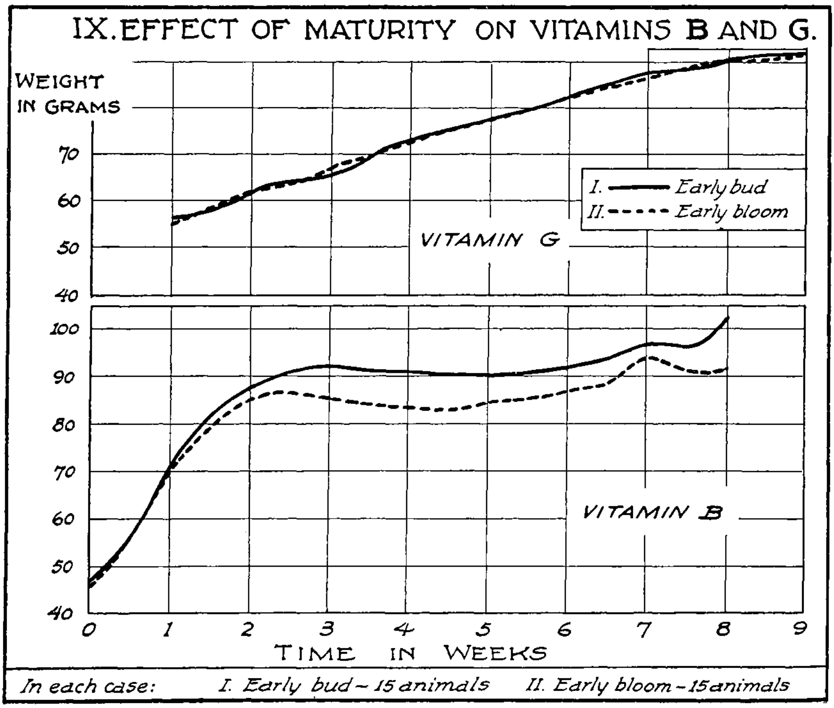
It was unfortunate for our study of the vitamin-B content that there were available for comparison hays of only two stages of

Table 18.—Biological Assay—Vitamin B—Alfalfa Hays, Crop of 1930—Effect of Maturity—Cured in Diffused Light.

Sample No.	Stage of Growth	Cutting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Consumed
3192	Early Bud	First	5	.50 g.	49.4	121.8	9.0 g.	6.0	56	458
3203	Early Bud	Second	5	.50	46.2	112.0	8.2	5.5	56	464
3213	Early Bud	Third	5	.50	44.4	93.9	6.1	4.1	56	390
	Average				46.7	109.2	7.8	5.2	56	437
3193	Early Bloom	First	5	.50	49.8	110.2	7.5	5.0	56	462
3202	Early Bloom	Second	5	.75	44.5	118.7	9.2	4.1	56	469
3220	Early Bloom	Third	5	.50	45.3	76.6	3.9	2.6	53.8	338
	Average				46.5	101.8	6.9	3.9	55.2	423
3200	Late Bloom	Second	Not Tested							
3209	Late Bloom	Second	5	.25	49.0	91.2	5.2	7.0	56	379

maturity for each of the three crops. Had a late-bloom stage been available in each crop, our data would doubtless have been more conclusive. However, so far as complete data were available, they indicated for all three crops that there was a gradual diminution of vitamin B up to the early bloom stage. Altho these results were consistent, the differences apparently were not large enough to be significant according to statistical tables appended to this paper. The consistency of the results is manifest both from Table 18 and the growth curve (Chart IX). We believe that these results would have shown to better advantage had our animals been depleted of vitamin B before applying the tests.

An anomaly in the table is shown by sample No. 3209, since it indicated more vitamin B in the mid-bloom stage of the second crop than was present in the less-mature stages.



EFFECT OF SUN CURING VS. DIFFUSED LIGHT

Perhaps the most striking results of our vitamin studies were those showing the adverse effect of drying in direct sunlight upon the vitamin-B content of our alfalfa hays. This study was suggested by some published work from the Kansas Experiment Station (26(b) and elsewhere. It seemed unnatural to believe that the direct rays of the sun should tend to lower the vitamin content of alfalfa; nevertheless, this is what our data consistently indicated.

The differences revealed by the rat-feeding tests were, in our minds, of significant order altho our statistical criteria did not indicate this. We ascribe this lack of confirmation principally to the fact that our animals were not depleted, but also because more than five animals to a test would have been desirable.

In the two series of homologous samples cited, each consisting of five hays, every sample dried in diffused light contained appreciably more of vitamin B than its homologue dried in the sun. The composite graph (Chart X) as well as Table 19 show this difference, it seemed to us, in a very convincing manner.

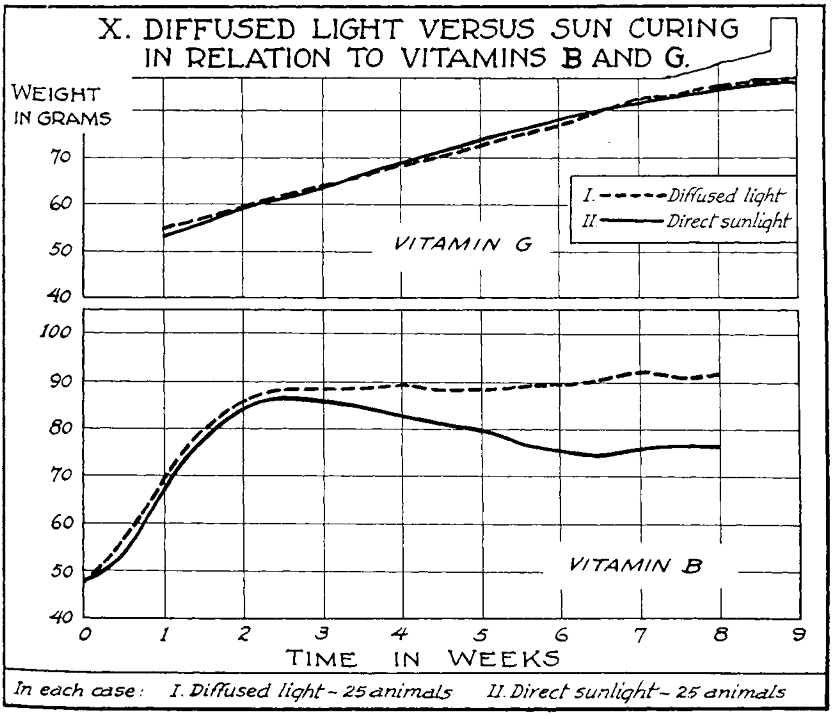


Table 19.—Biological Assay—Vitamin B—Alfalfa Hays, Crop of 1930—Sun-cured vs. Diffused Light.

Cured in Diffused Light

Sample No.	Stage of Growth	Cut-ting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Con-sumed
3213	Early Bud	Third	5	.50 g.	44.4	93.9	6.2	4.1	56	390 g.
3193	Early Bloom	First	5	.50	49.8	110.2	7.5	5.0	56	462
3202	Early Bloom	Second	5	.75	44.5	118.7	9.3	4.1	56	469
3220	Early Bloom	Third	5	.50	45.3	76.6	3.9	2.6	53.8	338
3209	Late Bloom	Second	5	.25	49.0	91.2	5.2	7.0	56	379
	Average				46.6	98.1	6.4	4.6	55.5	408

Cured in Direct Sunlight

Sample No.	Stage of Growth	Cut-ting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Con-sumed
3214	Early Bud	Third	5	.50 g.	50.0	80.0	3.7	2.5	55.6	395
3194	Early Bloom	First	5	.50	46.0	72.8	3.1	2.0	52.6	325
3201	Early Bloom	Second	5	.50	49.4	80.9	3.9	2.6	55.8	364
3221	Early Bloom	Third	5	.50	43.1	63.6	2.6	1.7	52.0	324
3210	Late Bloom	Second	5	.25	47.0	74.6	3.4	4.6	56.0	372
	Average				47.1	74.4	3.4	2.7	54.4	356

EFFECT OF THE SEASON'S PROGRESSION ON THE QUALITY OF ALFALFA HAY

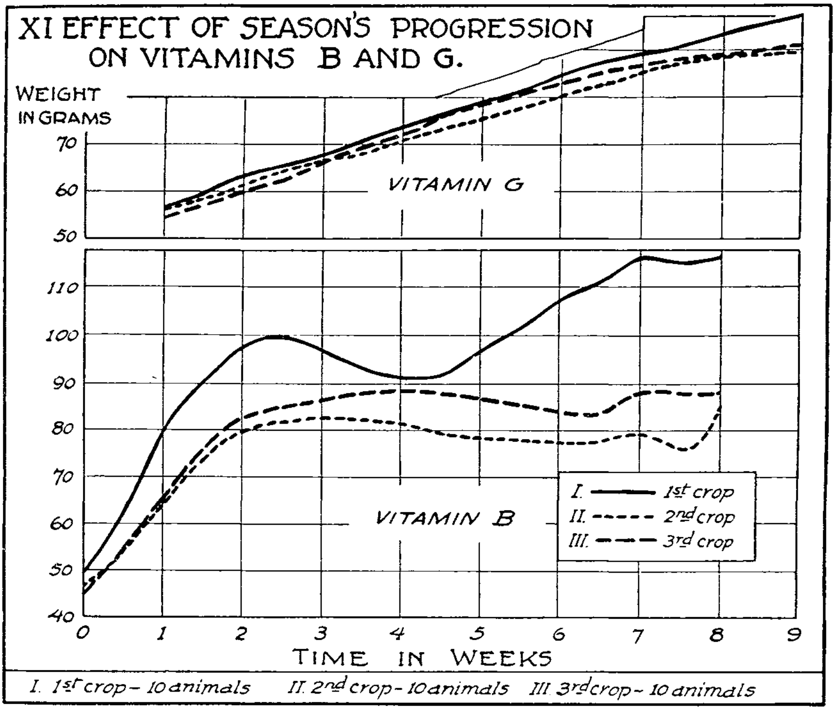
In Northeastern Colorado, where much stock feeding is carried on, it has been observed that hay buyers generally prefer first-cutting alfalfa hay and then the other two crops in their normal sequence. It was with the idea of determining whether or not there exists a sound reason for this preference that the following study was made.

We took hays representing two stages of maturity for each of our customary three crops, to make the chemical as well as the biological assay comparisons.

In the face of the analyses (Table 4) it was difficult to account for the results of our feeding tests. The results as set forth in Table 20 and Chart XI are quite regular and consistent in showing a gradual diminution of vitamin-B content with the progress of the growing season. Altho the differences are not large our statistical figures show them to be of significant order.

Table 20.—Biological Assay—Vitamin B—Alfalfa Hays, Crop of 1930—Effect of Season's Progression.

Sample No.	Stage of Growth	Cut-ting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Con-sumed
3192	Early Bud	First	5	.50 g.	49.4	121.8	9.0	6.0	56	458 g.
3193	Early Bloom	First	5	.50	49.8	110.2	7.5	5.0	56	462
	Average				49.6	116.0	8.3	5.5	56	460
3203	Early Bud	Second	5	.50	46.2	112.0	8.2	5.5	56	464
3202	Early Bloom	Second	5	.75	44.5	118.7	9.3	4.1	56	469
	Average				45.3	115.3	8.7	4.8	56	466
3209	Late Bloom	Second	5	.25	49.0	91.2	5.3	7.0	56	379
3213	Early Bud	Third	5	.50	44.4	93.9	6.2	4.1	56	390
3220	Early Bloom	Third	5	.50	45.3	76.6	3.9	2.6	53.8	338
	Average				44.8	85.2	5.0	3.3	54.9	364



The apparent inconsistency between the curves in vitamin B above and the results in Table 20 arises from the higher level of alfalfa fed, the second cutting being fed on a higher level.

EFFECT OF MOLDINESS ON THE QUALITY OF ALFALFA HAYS

It is long and well known to stock feeders that moldiness is a very undesirable quality in any food product. For purposes of discussion, it is logical to believe that the damage of molds may fall into two categories. On the one hand, the growth of molds may merely cause a dissipation or consumption of the nutrient values of a given food product. Again, if not actually destroyed, the proteins and other ingredients may be changed into other compounds which may be toxic to animals. The undesirable effects may be a combination of both of the above factors and perhaps even others not here mentioned.

One is handicapped in attempting to evaluate exactly the degree of damage done to alfalfa by molds because there may be several molds taking part and also because there is no exact method for determining the degree of moldiness. Nevertheless, we grouped the analyses of some of our samples which were known to be moldy to see what deductions, if any, could be made.

From the viewpoint of biological tests (Table 21 and Chart XII) some deductions seemed to us permissible.

While the vitamin-B content was somewhat diminished even by a mild degree of moldiness, the damage was not serious.

It is significant, we believe, that the two hays most moldy by appearance (in our collection Nos. 3227 and 3211) should also be almost depleted of their vitamin-B content.

Table 21.—Biological Assay—Vitamin B—Alfalfa Hays, Crop of 1930—Effect of Molds.

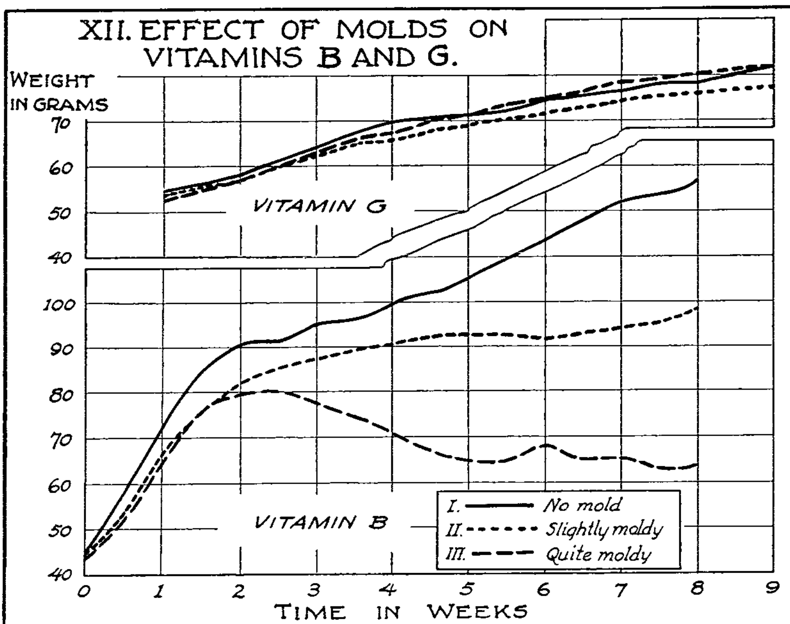
Sample No.	Stage of Growth	Rain Inches	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Consumed
*3230	E. Bl. (Swath)	No	5	.50 g.	47.4	127.2	9.9	6.6	56	452
3231	E. Bl. (Swath)	1.5	5	.50	41.6	96.1	6.8	4.5	56	392
3212	E. Bl. (Swath)	4.0	5	.50	47.8	102.8	6.9	3.0	56	441
3232	E. Bl. (Cock)	1.0	5	.50	45.0	97.4	6.5	4.3	56	415
3233	E. Bl. (Cock)	2.0	5	.50	44.8	100.0	6.9	4.6	56	419
3227	E. Bl. (Cock)	2.0	5	.75	44.2	53.8	1.2	0.5	43.8	284
3211	E. Bl. (Cock)	4.0	5	.50	43.3	61.2	2.2	1.0	48.6	298

* 3230 was a check sample of non-moldy hay.

COMPARISON OF COLORADO HAYS WITH HAYS FROM OTHER STATES

In 1931 we had shipped to us several samples of alfalfa hay from Eastern and Southern States for the purpose of making comparisons with Colorado hays.

In the states mentioned, the rainfall is much heavier than in Colorado and hay is much more likely to be damaged in the field than is the case in our state. However, none of the foreign samples obtained had been rained upon while curing, according to our information regarding these samples.

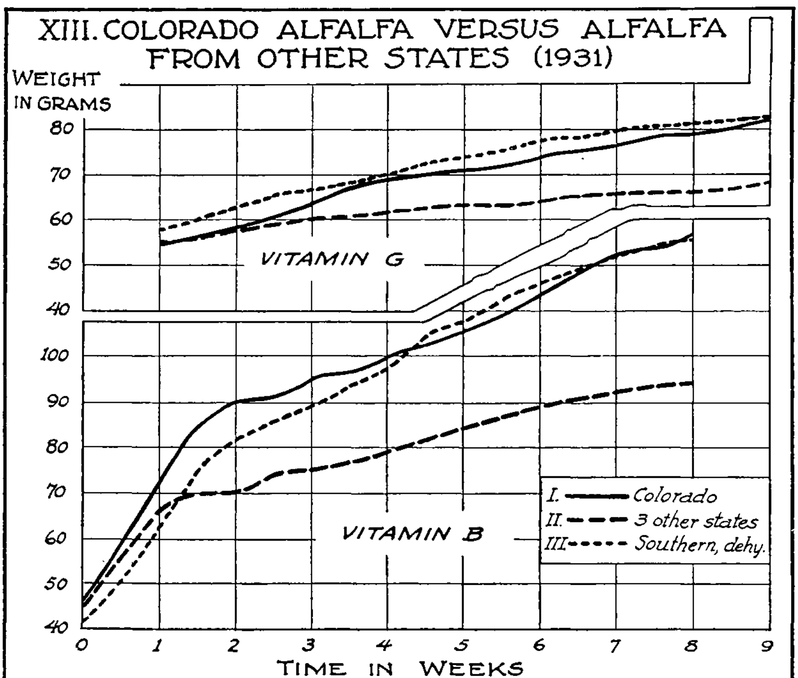


I. No mold - check sample — 5 animals. II Slightly moldy - 4 samples — 20 anim. III. Quite moldy - 2 samples — 10 animals.

These comparisons (Table 22 and Chart XIII) were carried out in accordance with all of our previous work. It may be seen from the tabulation that the vitamin-B content varied considerably. With one exception, the Colorado control sample showed to advantage, altho all of the foreign hays were perhaps of average quality with respect to vitamin B. The one exception was a mechanically dried sample from a Southern State (No. 3241). The two samples Nos. 3241 and 3242 represent one and the same hay except that the first was dried when fresh cut by use of a mechanical dryer, while the second was dried naturally in the field.

Table 22.—Biological Assay—Vitamin B—Comparison of Colorado Alfalfa Hays with Hays from other States.

Sample No.	Location	Cutting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Life Days	Food Consumed
3230	Fort Collins....	Third	5	.50 g.	47.4	127.2	9.9	6.6	56	452
3234	Eastern..........	First	5	.50	43.0	97.4	6.7	4.5	56	393
3235	Eastern..........	Second	5	.50	45.6	110.4	8.1	5.4	56	439
3236	Southern A......	Second	5	.50	46.8	118.6	8.9	5.9	56	451
3237	Southern A......	Third	5	.50	45.4	94.8	6.1	4.1	56	399
3238	Southern A......	Third	5	.50	44.4	107.6	7.9	5.2	56	433
3241	Southern B......	Dryer	5	.50	41.8	126.4	10.5	7.0	56	482
3242	Southern B......	(field cured)	5	.50	46.6	98.4	6.4	4.3	56	428



I. Good Colorado alfalfa – 5 animals. II. 6 alfalfas-other states – 30 animals.
III. Southern alfalfa, dehydrated – 5 animals

SUMMARY AND CONCLUSIONS

In collecting the data presented in this study of the vitamin-B content of alfalfa hays, in excess of 400 rats were fed in individual cages. When the approximately correct feeding level was established, each of the hays under investigation was fed to five or more animals. The animals were not previously depleted of their body store of vitamin B, but were placed directly on test at the age of 28 days and the tests were carried over an 8-week period. Altho later work by other authors and that in our own laboratory indicates the desirability of depletion of vitamin B for all experimental animals before placing them on test for vitamin-B content, yet we believe the data submitted represent a nearer approximation to the correct vitamin-B content of alfalfa hay than was shown in the available published literature, when this study was begun.

The studies represent a rather intensive investigation of Colorado alfalfas of one growing season (1930). However, the comparisons of our hays with those from other states represent samples of the season of 1931.

Whether the vitamin content of alfalfa varies from year to year still remains a question.

Our tests relating to the effect of the various methods of curing on the vitamin-B content, tho not exhaustive, indicate the following trends:

First, the effect of 1 inch of rainfall (and even up to 2 inches) did not seem to diminish but rather increased the vitamin-B content of alfalfa hay, provided it was quickly dried after receiving the shower.

Second, with only two stages of maturity (early bud and early bloom) represented, our data indicate a small diminution of vitamin B with the increasing age of the plant.

Third, in every comparison made, samples of identical hays dried in diffused light gave a very appreciably higher vitamin-B content than those dried in direct sunlight.

Fourth, that the vitamin-B content in alfalfa hay does decrease with the progression of the growing season, is indicated by our tests. The three crops are aligned in the descending order of vitamin-B content as crop 1, crop 2 and crop 3.

Fifth, excessive molding of the hay in the curing process, does decidedly diminish the vitamin-B content, as indicated by our comparisons.

Sixth, Colorado alfalfa hay showed up to advantage over some hays from other states in vitamin-B content, altho these foreign hays were of average quality and showed no particular lack of this factor.

And finally, on the basis of several hundred animals studied, we feel justified in saying that either the vitamin-B factor itself or some other ingredient in alfalfa hay did adversely affect the kidney and bladder tract of numerous tho not by any means all of the animals studied. Many of the animals, while continually gaining in weight, showed this effect.

We may also add that, while our test animals throve fairly well on many of our average-quality hays, even while gaining in weight, some showed evidence of polyneuritis, which would indicate that vitamin B might be supplemented to alfalfa hay feed to good advantage.

VITAMIN-G STUDIES

In the literature reviewed dealing with vitamins in alfalfa, only one article by McCollum and Simmonds (14) referred to studies of vitamin G in alfalfa seed and other seeds.

The technical studies dealing with the distinction between B and G-vitamin factors in foods have progressed so far in recent years that we felt it would be advisable as well as safe to make a study of the G factor in alfalfa hay. It seemed the more advisable since the hay samples were all prepared, and the opportunity once rejected might not return as favorably for such studies. After working out a successful basal G-free diet, these studies were carried out in like manner and upon the same alfalfa samples as used in the previous tests for vitamins A and B.

NEGATIVE CONTROLS.—A total of 12 negative controls with an average initial weight of 46.9 grams attained to an average maximum weight of 53.1 grams at the point of depletion. See Chart VII.

As shown in Table 23, this point was reached at from 6 to 9 days, with an average depletion period of about 7.5 days. The depletion ratio of these animals according to the above figures was 1.13, indicating a very small storage of vitamin G in rats 28 days old. Their average final weight was 44.0 grams at the end of 61 days or an average of 2.9 grams below their initial weight. In contrast with our negative B controls it is noteworthy that of these 12 and a much larger total number of animals used in preliminary tests, only two deaths were registered on our basal G-free Diet 17.

Table 23.—Control Animals—Vitamin G (1930-31)—Semi-Weekly Gain Chart (weight in grams).

No. of Animals	Legend																			
12	Neg........	46.9	50.0	53.1	52.9	52.6	52.3	51.8	50.9	50.6	49.3	49.7	49.3	48.0	47.1	46.4	45.3	44.0	44.0	44.0
12	Pos........	47.0	57.5	68.6	79.5	89.6	97.1	104.0	112.0	119.0	127.2	133.1	142.9	148.0	155.2	159.9	166.0	167.0	173.5	174.7

Fate of the Animals

No. of Animals	Legend	Initial Weight	Maximum Weight	Final Weight	Days on Experiment	Food Consumed	Animals Died	Average Weekly Gain
12	Negative....................	46.9	53.1	44.0	59.7	284.0	2	–.3 g. } 9 week exp.
12	Positive........................	47.0	174.7	174.7	63.0	709.0	None	14.2 g. } 9 week exp.

POSITIVE CONTROLS.—The history of our positive controls may be summed up in a few words. Of 12 animals with an average initial weight of 47.0 grams, all gained regularly to an average maximum of 175.7 grams at the end of 63 days. This represented an average weekly gain of 14.2 grams and the animals in all apparent respects were the equivalent of our young breeding stock. These animals had received our Diet 17 and water, plus 0.5 gram of yeast with 3 drops of cod liver oil daily.

RESULTS OF VITAMIN-G STUDIES.—All animals were depleted before being placed on experiment for vitamin-G measurement. A casual observation of the data collected under the vitamin-G studies would warrant the statement, we believe, that the G factor in alfalfa hays of Colorado is far less susceptible to wide fluctuations than are either the A or B factors. This might reasonably be expected from the fact that vitamin G represents the heat-stable portion of the former vitamin-B complex.

The trends indicated by our tabulated data were so small in most cases as to fall easily within the limits of experimental error. On the other hand, tho small, they were so consistent in many instances that it was believed they should not be ignored.

Table 24.—Biological Assay—Vitamin G—Alfalfa Hays, Crop of 1930—Effect of Rain—Cured in Direct Sunlight.

Sample No.	Stage of Growth	Cutting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. G	Life Days	Food Consumed 63 Days
No Rain										
3194	Early Bloom	First	5	.35 g.	57.2	99.2	5.2 g.	5.0	56	422
3201	Early Bloom	Second	5	.35	57.6	87.4	3.7	3.5	56	400
3221	Early Bloom	Third	5	.35	54.4	85.8	3.9	3.7	56	403
3214	Early Bud	Third	5	.35	52.0	82.8	3.8	3.6	56	398
	Average				55.3	88.8	4.2	4.0	56	406
3210	Late Bloom	Second	5	.35	52.0	78.8	3.3	3.2	56	339
1 Inch Rain										
3196	Early Bloom	First	5	.35	56.0	86.4	3.8	3.6	56	415
3205	Early Bloom	Second	5	.35	52.2	83.4	3.9	3.7	56	386
3223	Early Bloom	Third	5	.35	50.8	86.4	4.5	4.2	56	411
3216	Early Bud	Third	5	.35	55.0	93.4	4.8	4.6	56	437
	Average				53.5	87.4	4.2	4.0	56	412
2 Inch Rain										
3199	Early Bloom	First	5	.35	56.8	88.8	4.0	3.8	56	387
3208	Early Bloom	Second	5	.35	55.2	92.8	4.7	4.5	56	383
3225	Early Bloom	Third	6	.35	48.8	80.1	3.9	3.7	56	385
3218	Early Bud	Third	5	.35	53.8	93.8	5.0	4.7	56	424
	Average				53.6	88.8	4.4	4.2	56	395
3227	E. Bl. (Cock)	Third	6	.35	49.8	80.0	3.8	3.6	56	400
4 Inch Rain										
3211	In Cock	Second	5	.35	55.6	83.6	3.5	3.3	56	414
3212	In Swath	Second	6	.35	55.1	83.1	3.5	3.3	56	413

EFFECT OF RAIN ON VITAMIN-G CONTENT.—Table 24 as well as Chart VIII show some rather erratic effects upon the G content of hay, caused by rain during the curing process. In the first crop an appreciable loss was noted and that loss was greater for a 1-inch than for a 2-inch rain. In the second crop appeared an anomalous effect of a progressive increase of vitamin G with the increase of rainfall.

This was, however, in accord with the behavior of vitamin B in the second alfalfa crop. The third crop showed a small gain in vitamin G for a 1-inch rain and approximately no loss for a 2-inch rain. The same hay (No. 3227), cocked when partly dry, tho it had been thoroly soaked by a 2-inch rain, showed only a slight loss of vitamin G.

While our basic data for the two extraneous samples (Nos. 3211 and 3212) were incomplete, these had received 4 inches of natural rain and it is apparent from the table that even this large amount of moisture did not seriously diminish the vitamin-G content.

A general comparison of the effects of rain upon the vitamins B and G in our alfalfa hays would indicate that a 1-inch rain upon partly dry alfalfa was beneficial rather than harmful to the resultant hay. This would be true with the proviso that after wetting the hay was subsequently dried rapidly to prevent the formation of molds. Larger increments of rain would diminish the vitamin-B content, while the G would be affected in a much lesser degree, if at all.

EFFECT OF MATURITY OF THE PLANT.—Next, our comparison was carried out with regard to the effect upon the G content in the plant, depending upon the age at which the plant was cut.

Table 25 and Chart IX show for the second crop a steady but small decrease of the G factor with age. This observation, however, was not borne out by the first and third crops, which, as far as data were available, show increases in vitamin G from the early bud to the early bloom stage. It was unfortunate that samples were not available representing the late-bloom stage of the first and third crops, to complete the table.

EFFECT OF DIFFUSED LIGHT VS. SUN CURING.—Of the five hays compared for the effect of sunlight versus diffused light in drying (Table 26 and Chart X), the results appeared rather inconclusive.

Three of the samples showed slight losses of vitamin G in the sun curing, while one was neutral and one showed a gain. These five comparisons are far from adequate to warrant any general conclusions. We merely present them as obtained, without comment, especially since our statistical criteria on this table are also neutral.

One thing was certainly indicated as a result of these tests, it being the fact that curing in direct sunlight does not affect the vitamin-G content to the extent that it affects vitamin B.

EFFECT OF SEASON'S PROGRESSION ON VITAMIN G.—Altho there was no apparent reason why the vitamin content of alfalfa hays should be affected by the mere fact that they were grown in the early spring or in the heat of summer or again in the cool of approaching autumn, nevertheless, we had gone to some trouble to prepare our hay samples and since these were at hand, it was thought advisable to carry out such comparisons.

Table 20, representing the study of vitamin B in this respect, showed a small but consistent diminution of that factor in each succeeding crop. A comparison of the same hays with respect to vitamin G, as shown in Table 27 and Chart XI, revealed some very small differences.

Table 25.—Biological Assay—Vitamin G—Alfalfa Hays—Crop of 1930—Effect of Maturity—Cured in Diffused Light.

Sample No.	Stage of Growth	Cutting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. G	Life Days	Food Consumed 63 Days
3192	Early Bud	First	5	.35 g.	58.4	95.0	4.5	4.3	56	401
3203	Early Bud	Second	5	.35	56.8	92.8	4.5	4.2	56	388
3213	Early Bud	Third	6	.35	54.6	89.2	4.3	4.1	56	402
	Average				56.6	92.3	4.4	4.2	56	397
3193	Early Bloom	First	5	.35	55.2	100.0	5.6	5.3	56	407
3202	Early Bloom	Second	5	.35	56.6	85.8	3.6	3.5	56	385
3220	Early Bloom	Third	6	.35	54.1	91.3	4.6	4.4	56	424
	Average				55.3	92.3	4.6	4.4	56	405
3209	Late Bloom	Second	5	.35	45.4	64.4	2.4	2.2	56	367

Table 26.—Biological Assay—Vitamin G—Alfalfa Hays, Crop of 1930—Sun-cured vs. Diffused Light—Cured in Diffused Light.

Sample No.	Stage of Growth	Cutting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. G	Life Days	Food Consumed 63 Days
3213	Early Bud	Third	5	.35 g.	54.6	89.2	4.3	4.1	56	402
3193	Early Bloom	First	5	.35	55.2	100.0	5.6	5.3	56	407
3202	Early Bloom	Second	5	.35	56.6	85.8	3.6	3.5	56	385
3220	Early Bloom	Third	6	.35	54.1	91.3	4.6	4.4	56	424
3209	Late Bloom	Second	5	.35	45.4	64.4	2.3	2.2	56	367
	Average				53.2	86.1	4.1	3.9	56	397
	Cured in Direct Sunlight									
3214	Early Bud	Third	5	.35	52.0	82.8	3.8	3.6	56	398
3194	Early Bloom	First	5	.35	57.2	99.2	5.2	5.0	56	422
3201	Early Bloom	Second	5	.35	57.6	87.4	3.7	3.5	56	400
3221	Early Bloom	Third	5	.35	54.4	85.8	3.9	3.7	56	403
3210	Late Bloom	Second	5	.35	52.0	78.8	3.3	3.2	56	339
	Average				54.6	86.8	4.0	3.8	56	392

The G contents of all three crops when cut in the early bud (immature) stage do not indicate a difference of a magnitude which could be considered significant.

Alfalfas harvested in the early bloom stage (which constitutes normal practice in Northern Colorado) indicated that the first and third crops contain the highest amounts of the G factor, while the midsummer crop lags behind. According to our criteria it is doubtful that these differences were sufficiently large to be significant.

EFFECT OF MOLDS ON VITAMIN-G CONTENT.—Table 28 and Chart XII are comparisons of a group of our hays which were moldy as a result of wetting. We tried to arrange the samples in logical sequence, starting with a non-moldy check sample (No. 3230) at the top and ending with (No. 3211), a sample which was to all appearances the most moldy of all our hays. The degree of moldiness was difficult to determine and the results appear rather erratic. Some of the samples apparently lost vitamin G, but the most significant conclusion from this table is that the two samples receiving the most thoro drenching (4 inches of rain) seemed to have suffered the least losses of vitamin G. In a measure this falls in line with previous statements regarding the stability of the vitamin-G factor.

Table 27.—Biological Assay—Vitamin G—Alfalfa Hays, Crop of 1930—Effect of Season's Progression.

Sample No.	Stage of Growth	Cut-ting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gains	Units per g. Vit. G	Life Days	Food Con-sumed 63 Days
3192	Early Bud........	First	5	.35 g.	58.4	95.0	4.6	4.3	56	401
3193	Early Bloom....	First	5	.35	55.2	100.0	5.6	5.3	56	407
	Average............	....	....	....	56.8	97.5	5.1	4.8	56	404
3203	Early Bud........	Second	5	.35	56.8	92.8	4.5	4.3	56	388
3202	Early Bloom....	Second	5	.35	56.6	85.8	3.7	3.5	56	385
	Average............	....	....	....	56.7	89.3	4.1	3.9	56	386
3209	Late Bloom......	Second	5	.35	45.4	64.4	2.4	2.3	56	367
3213	Early Bud........	Third	5	.35	54.6	89.2	4.3	4.1	56	402
3220	Early Bloom....	Third	6	.35	54.1	91.3	4.7	4.4	56	424
	Average............	....	....	....	54.3	90.2	4.5	4.2	56	413

Table 28.—Biological Assay—Vitamin G—Alfalfa Hays, Crop of 1930-31—Effect of Molds.

Sample No.	Stage of Growth	Rain Inches	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. G	Life Days	Food Con-sumed 63 Days
3230*	E. Bl. (Swath)	No	5	.35 g.	54.4	82.2	3.5	3.3	56	404
3231	E. Bl. (Swath)	1.5	5	.35	54.6	79.4	3.1	2.9	56	408
3212	E. Bl. (Swath)	4.0	6	.35	55.1	83.1	3.5	3.3	56	413
3232	E. Bl. (Cock)	1.0	5	.35	55.6	80.2	3.1	2.9	56	416
3233	E. Bl. (Cock)	2.0	5	.35	50.6	66.8	2.0	1.9	56	371
3227	E. Bl. (Cock)	2.0	6	.35	49.8	80.0	3.8	3.6	56	400
3211	E. Bl. (Cock)	4.0	5	.35	55.6	83.6	3.5	3.3	56	414

* Check sample, non-moldy.

COMPARISON OF COLORADO ALFALFA WITH THAT FROM OTHER STATES.—A comparison of Colorado alfalfa with that from several other states, with respect to vitamin-G content, was carried out as in the cases of vitamins A and B.

One Colorado sample of good-quality hay was compared with seven foreign samples, as set forth in Table 29 and Chart XIII.

The comparison is neither an exhaustive nor a critical study, since an insufficient number of samples are involved. The comparison, however, brought out at least one valuable indication with respect to vitamin G. A content of 3.5 units of vitamin G is not exceptionally high for Colorado alfalfa, some of our samples containing as high as 5.0 units. Yet the Colorado sample used for comparison is favored in G content above all of the foreign hays. It was difficult to understand why the vitamin-G content of these foreign hays should be so low; the more so because the many samples involved in our previous work with this factor indicated in all Colorado hays tested that the vitamin-G content was moderately high, rather uniform and relatively stable against the exigencies of the curing process. At any rate, of the vitamins studied, it was the least susceptible to wide fluctuations under adverse treatment.

SUMMARY AND CONCLUSIONS

During the course of our study of Colorado alfalfas of the 1930 season it was observed that the second crop was lower in both vitamins B and G. In years when our third crop is permitted to attain the early bloom stage of growth before frosts, that crop is usually second in nutrient value of the three crops. In attempting to account for this fact we have been inclined to ascribe the relative inferiority of the second crop to the heat of midsummer, with excessive transpiration of moisture, and the consequent woodiness or lesser succulence of that crop.

Table 29.—Biological Assay—Vitamin G—Colorado Alfalfa Hay Compared with Hays from Other States.

Sample No.	Location	Cutting	No. of Rats	Level Fed	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. G	Life Days	Food Consumed 63 Days
3230	Ft. Collins	Third	5	.35 g.	54.4	82.2	3.5	3.3	56	404
3234	Eastern	First	5	.35	57.0	61.8	0.6	0.6	56	375
3235	Eastern	Second	5	.35	54.0	65.4	1.4	1.4	56	372
3236	Southern A	Second	5	.35	55.4	68.0	1.6	1.5	56	364
3237	Southern A	Third	5	.35	55.4	59.2	0.5	0.4	54.6	346
3238	Southern A	Third	5	.35	53.0	75.8	2.8	2.7	56	370
3241	Southern B	(Dryer)	5	.35	58.0	82.8	3.1	3.0	56	432
3242	Southern B	(Field Cured)	5	.35	55.6	76.2	2.6	2.4	56	389

It was also casually observed in making comparisons between B and G-vitamin values of our hays that sometimes when the B value diminished as a result of adverse treatment, the G value would increase. However, the scope of our present work was not exhaustive enough to warrant any specific conclusion regarding this point.

The most we can say regarding the effect of harsh curing treatments upon the vitamin-G content of alfalfa hay is, that this factor suffers least, if at all; that it is rather stable and uniform in distribution in our Colorado hays and that this content ranges from about 3 to 5 units per gram in amount for good Colorado hays.

VITAMIN C

The conclusions drawn from the vitamin-C determinations can be summed up very briefly.

Green alfalfa has an abundance of vitamin C as evidenced by the continued good health of the guinea pigs used in this work, but vitamin C is lost when the alfalfa is cut and cured. The guinea pigs would eat the freshly cured alfalfa until scurvy had developed to such an extent that they were unable to chew the hay.

There is practically no vitamin C in alfalfa hay, which confirms other investigations in this field.

VITAMIN D

Since other workers have found that either irradiation of the animals or irradiation of the food prevents rickets, we did not determine vitamin D in our hays, assuming that, with the abundant sunshine in Colorado, this vitamin is probably fairly well supplied.

THE NECESSITY OF DEPLETION IN THE DETERMINATION OF VITAMIN B

As stated earlier in this paper, our data for vitamin-B determinations were collected on the basis of non-depleted animals. The work was based largely upon alfalfa hays harvested from a given plot on our experimental farm during the season of 1930. The first determinations of vitamin B were made about 6 months after the harvest and were made on the non-depletion basis.

Table 30.—Comparison of Data from Depleted and Non-depleted Animals in Determination of Vitamin B in Alfalfa Hays.

Sample No.	No. of Rats	Level Fed in Grams	Initial Weight	Final Weight	Weekly Gain	Units per g. Vit. B	Units B Interpolated	Weekly Food	Days of Life
A.-1931-Non-depleted									
3192	5	.5	49.4	121.8	9.0	6.0	3.3	57.2	56
3193	5	.5	49.8	110.2	7.5	5.0	2.3	57.7	56
3209	5	.25	49.0	91.2	5.27	7.0	1.7	47.7	56
3220	5	.50	45.3	76.6	3.9	2.6	.2	42.2	53.8
B.-1933-Depleted									
3192	5	.66	70.4	98.4	3.5	1.7	....	39.7	56
3193	5	.66	68.0	87.4	2.4	1.2	....	36.7	56
3209	5	.66	76.0	93.8	2.2	1.1	....	38.9	56
3220	5	.66	72.4	81.4	1.1	0.5	....	35.7	55.6

In the spring of 1933, about 2.5 years after the first tests, we selected four representative samples of the identical hays and repeated the vitamin-B tests except that this time we depleted our animals. The four samples represented in Table 30 had been stored in a dry, cool condition in closed tin cans.

Table 30 represents an attempt to check back on those samples upon the basis of depletion of the animals before applying the test, and also to determine the fate of vitamin B under the optimum conditions of storage as stated above.

It is interesting to note that 20 negative controls in the work of 1931 showed a depletion ratio of 1.65 over the initial weight of the animals. In the tests of the spring of 1933 the 20 animals used in the actual determinations by pre-depletion gave us a depletion ratio of 1.61.

In section A of Table 30, we attempted by interpolation on the basis of a depletion ratio of 1.65, to reach a figure nearer the actual vitamin-B content per gram of hay than that expressed on the non-depletion basis. While this gave only hypothetical figures, it served to explain away the apparent anomaly of sample 3209, which in Table 18 tended to confound our results and deductions by such an inordinately high B content as 7 units in a mature (half bloom) stage of plant growth.

Section B of Table 30 (if we may use the hypothetical figures for units per gram of vitamin B as recalculated) would indicate that in 2 years of storage these samples had deteriorated to the extent of about 50 percent if we overlook the figures for sample 3220, which results are so small as to fall far within limits of experimental error.

STATISTICAL EXAMINATION*

The large number of disturbing influences which cannot be controlled in vitamin determinations has led to the use of statistics in the examination of experimental results. The tables given here rank the hays of each group in order of vitamin content and also according to what differences are significant. A significant difference means that the difference between the two samples of hay is greater than the amount which could reasonably be due to chance factors. Therefore where a significant difference can be found in the growth of the rats and consequently in the vitamin content of the hays, it was due to actual variations in the alfalfa.

The statistical method used in the computation of significant differences and in ranking the hays was as follows:

I. The hays in the group were arranged in a column of descending vitamin content. For each hay the arithmetical mean of

*We wish to acknowledge our indebtedness to Mr. Earl Balis for making the calculations shown in the statistical tables.

the vitamin content as determined by individual rats, was calculated. Since a homogeneous group of rats is desirable for best results, only the data on rats that lived the average life of the group were used. Those that died sooner were discarded as exceptional cases. This accounts for certain discrepancies between the original and the statistical tables since originally every rat was included in the computation.

II. The standard deviation from the mean was determined by application of the following formula:—

$$S.D._n \text{ (standard deviation)} = \frac{\text{sum of the squares of individual deviations}}{\text{number of cases}}$$

III. The probable errors were calculated from the standard deviation. $P.E._n$ (probable error of the mean) $= \frac{0.6745 \times S.D.}{\sqrt{\text{Number of cases}}}$

$P.E._d$ (probable error of the difference between two means) $= \sqrt{(P.E._{m_1})^2 + (P.E._{m_2})^2}$

IV. If the difference between two means was greater than $3 \times P.E._d$ it was considered significant since it is 95.8 percent sure that this difference is a true one.

V. The hays arranged in the column were bracketed together in groups which showed no significant differences; from this diagram they were ranked in the following manner:—

Hay	Units A/.1g	Rank
41	6.8	1
42	2.8	2
35	2.4	3
38	2.0	4

There was a significant difference between "41" and "42," none between "42" and "35;" a difference between "42" and "38" but none between "35" and "38." This divided the hays into three groups:

Group 1........................Hay "41"
Group 2........................"42" and "35"
Group 3........................"35" and "38"

Hay "41" was, of course, given the rank of 1. Since "42" belonged in Group 2 with "35" but not in the lower group (those where "35" is also found), hay "42" was ranked higher than "35" even tho, if considered alone, these two hays would show no significant difference. By similar reasoning "25" is inferred to be superior to "38" because "35" belongs in both Groups 2 and 3.

Table 31.—Statistical Examination of Tables 10 and 11-A—Effect of Rain on Vitamin A—Sun-cured.

Sample No.	Stage of Growth	Cutting	Treatment	Level Fed in g.	No. of Rats	Vit. A in .1 g. Hay	Probable Error	(P.E.)²	Rank
3194	Early Bloom	First	No Rain	.05	9	3.6	.270	.0729	1
3196	Early Bloom	First	1" Rain	.05	8	2.0	.056	.0031	2
3199	Early Bloom	First	2" Rain	.20	8	1.0	.128	.0164	3
3201	Early Bloom	Second	No Rain	.05	6	1.8	.243	.0590	1
3205	Early Bloom	Second	1" Rain	.10	9	1.8	.272	.0740	1
3208	Early Bloom	Second	2" Rain	.10	9	1.4	.285	.0812	1
3214	Early Bud	Third	No Rain	.05	10	4.2	.200	.0400	1
3216	Early Bud	Third	1" Rain	.05	10	3.2	.179	.0320	2
3218	Early Bud	Third	2" Rain	.10	8	1.6	.207	.0428	3
3221	Early Bloom	Third	No Rain	.10	10	2.8	.185	.0342	1
3223	Early Bloom	Third	1" Rain	.20	10	1.3	.113	.0128	2
3225	Early Bloom	Third	2" Rain	.20	9	0.8	.110	.0121	3
3227	Early Bloom	Third	2" Rain (Cocked)	.10	9	2.2	.243	.0590	1

Table 32.—Statistical Examination of Table 11-B—Effect of Maturity on Vitamin A.

Sample No.	Stage of Growth	Cutting	Level Fed in g.	No. of Rats	Vit. A in .1 g. Hay	Probable Error	(P.E.)²	Rank
3192	Early Bud	First	.05	10	3.6	.100	.0100	2
3193	Early Bloom	First	.025	10	6.0	.634	.4019	1
3203	Early Bud	Second	.05	6	2.0	.575	.3306	2
3202	Early Bloom	Second	.05	10	3.2	.173	.0299	1
3209	Late Bloom	Second	.10	7	1.1	.316	.0998	3
3213	Early Bud	Third	.05	7	3.0	.379	.1436	2
3220	Early Bloom	Third	.025	10	6.4	.326	.1062	1

Table 33.—Statistical Examination of Table 11-C—Vitamin A in Sun-cured Hay Compared with Hay Cured in Diffused Light.

Sample No.	Stage of Growth	Cutting	Light Cured In	Level Fed	No. of Rats	Vit. A in .1 g. Hay	Probable Error	(P.E.)²	Rank
3193	Early Bloom	First	Diff.	.025	10	6.0	.634	.4019	1
3194	Early Bloom	First	Sun	.05	9	3.6	.270	.0729	2
3202	Early Bloom	Second	Diff.	.05	10	3.2	.173	.0299	1
3201	Early Bloom	Second	Sun	.05	6	1.8	.243	.0590	2
3220	Early Bloom	Third	Diff.	.025	10	6.4	.326	.1062	1
3221	Early Bloom	Third	Sun	.10	10	2.8	.185	.0342	2
3209	Late Bloom	Second	Diff.	.10	7	1.1	.316	.0998	1
3210	Late Bloom	Second	Sun	.10	9	1.5	.170	.0289	1
3213	Early Bud	Third	Diff.	.05	7	3.0	.379	.1436	1
3214	Early Bud	Third	Sun	.05	10	4.2	.200	.0400	1

Table 34.—Statistical Examination of Table 10—Seasonal Progression.

Sample No.	Stage of Growth	Cut-ting	Level Fed in g.	No. of Rats	Vit. A in .1 g. Hay	Probable Error	(P.E.)²	Rank
3192	Early Bud	First	.05	10	3.6	.100	.0100	1
3203	Early Bud	Second	.05	6	2.0	.575	.3306	3
3213	Early Bud	Third	.05	7	3.0	.379	.1436	2
3193	Early Bloom	First	.025	10	6.0	.634	.4019	1
3202	Early Bloom	Second	.05	10	3.2	.173	.0299	2
3220	Early Bloom	Third	.025	10	6.4	.326	.1062	1

Table 35.—Statistical Examination of Table 14—All Samples Third Cutting, Early Bloom—Effect of Mold on Vitamin A.

Sample No.	Level Fed in g.	No. of Rats	Vit. A in .1 g. Hay	Probable Error	(P.E.)²	Rank	Treatment
3230	.025	10	7.6	.744	.5535	1	Check-no rain
3232	.05	10	4.0	.235	.0552	2	1" rain in cock
3233	.05	9	4.0	.151	.0228	2	2" rain in cock
3229	.025	9	3.2	.931	.8867	3	Moldy in bell jar
3231	.05	9	2.2	.312	.0973	4	1.5" rain swath

Table 36.—Statistical Examination of Table 14—Vitamin A in Alfalfa Hays From Other States.

Sample No.	Origin	State of Growth	Cut-ting	Level Fed	No. of Rats	Vit. A in .1 g. Hay	Probable Error	(P.E.)²	Rank
3241	Southern B.	(Dehydrated)		.025	10	6.8	.545	.2970	1
3242	Southern B.	(field cured)		.05	9	2.8	.196	.0384	2
3235	Eastern	Early Bloom	Second	.10	5	2.4	.178	.0317	3
3238	Southern A.	Early Bloom	Third	.05	9	2.0	.310	.0961	4
3236	Southern A.	Early Bloom	Second	.10	10	1.0	.181	.0328	5
3234	Eastern	Early Bloom	First	.10	8	0.4	.327	.1069	6
3237	Southern A.	Full Bloom	Third	.20	7	0.3	.044	.0019	7

Table 37.—Statistical Examination of Table 17 and Effect of Rain on Vitamins B and G—Sun-cured.

				VITAMIN B						VITAMIN G (all fed .35 g. hay)			
Sample No.	Stage of Growth	Cut-ting	Treatment	Level Fed in g.	No. of Rats	Vit. B in 1 g. Hay	Probable Error	(P.E.)²	Rank	Vit. G in 1 g. Hay	Probable Error	(P.E.)²	Rank
3196	Early Bloom	First	1″ Rain	.5	5	5.7	.375	.1406	1	3.6	.489	.2391	2
3194	Early Bloom	First	No Rain	.5	5	2.1	.444	.1971	2	5.0	.240	.0576	1
3199	Early Bloom	First	2″ Rain	.5	5	3.5	.438	.1918	2	3.8	.139	.0193	2
3205	Early Bloom	Second	1″ Rain	.5	5	3.2	.378	.1429	1	3.7	.172	.0296	2
3201	Early Bloom	Second	No Rain	.5	5	2.6	.512	.2621	1	3.5	.192	.0392	3
3208	Early Bloom	Second	2″ Rain	.5	4	2.0	.450	.2025	1	4.5	.302	.0912	1
3216	Early Bud	Third	1″ Rain	.5	5	4.2	.560	.3136	1	4.6	.494	.2440	1
3214	Early Bud	Third	No Rain	.5	5	1.5	.506	.2560	2	3.7	.275	.0756	1
3218	Early Bud	Third	2″ Rain	.5	5	1.6	.326	.1063	3	4.8	.545	.2970	1
3223	Early Bloom	Third	1″ Rain	.5	5	1.3	.285	.0812	1	4.2	.412	.1697	1
3221	Early Bloom	Third	No Rain	.5	5	1.7	.352	.1239	1	3.8	.251	.0630	1
3225	Early Bloom	Third	2″ Rain	.75	5	1.9	.228	.0520	1	3.9	.422	.1781	1

Table 38.—Statistical Examination of Table 18 and Effect of Maturity on Vitamins B and G.

					VITAMIN B				VITAMIN G (all fed .35 g. hay)			
Sample No.	Stage of Growth	Cut-ting	Level Fed in g.	No. of Rats	Vit. B in 1 g. Hay	Probable Error	(P.E.)²	Rank	Vit. G in 1 g. Hay	Probable Error	(P.E.)²	Rank
3192	Early Bud	First	.5	5	6.0	.335	.1122	1	4.3	.196	.0380	1
3193	Early Bloom	First	.5	5	5.0	.408	.1664	1	5.3	.407	.1656	1
3203	Early Bud	Second	.5	5	5.5	.302	.0912	2	4.3	.207	.0428	1
3202	Early Bloom	Second	.75	5	4.1	.424	.1798	3	3.4	.251	.0630	2
3209	Late Bloom	Second	.25	5	7.0	.605	.3660	1	2.2	.292	.0852	3
3213	Early Bud	Third	.5	5	4.1	.315	.0992	1	4.0	.154	.0237	1
3220	Early Bloom	Third	.5	5	2.6	.628	.3944	1	4.2	.404	.1632	1

Table 39.—Statistical Examination of Table 19 and Vitamin B in Sun-cured Hay Compared with Hay Cured in Diffused Light.

Sample No.	Stage of Growth	Cutting	Light Cured In	Level Fed in g.	No. of Rats	VITAMIN B: Vit. B in 1 g. Hay	VITAMIN B: Probable Error	VITAMIN B: (P.E.)²	VITAMIN B: Rank	VITAMIN G (all fed .35 g. hay): Vit G in 1 g. Hay	VITAMIN G: Probable Error	VITAMIN G: (P.E.)²	VITAMIN G: Rank
3193	Early Bloom	First	Diffused	.50	5	5.0	.409	.1673	1	5.3	.407	.1656	1
3194	Early Bloom	First	Sun	.50	5	2.1	.444	.1971	2	5.0	.240	.0576	1
3202	Early Bloom	Second	Diffused	.75	5	4.1	.135	.0179	1	3.4	.251	.0630	1
3201	Early Bloom	Second	Sun	.50	5	2.6	.512	−.2621	2	3.5	.198	.0391	1
3220	Early Bloom	Third	Diffused	.25	5	2.6	.616	.3295	1	4.2	.404	.1632	1
3221	Early Bloom	Third	Sun	.25	5	1.7	.352	.1239	2	3.8	.251	.0630	1
3213	Early Bud	Third	Diffused	.50	5	4.1	.312	.0973	1	4.0	.154	.0237	1
3214	Early Bud	Third	Sun	.50	5	2.5	.506	.2560	2	3.7	.275	.0756	1
3209	Late Bloom	Second	Diffused	.50	5	7.0	.600	.3600	1	2.2	.292	.0852	2
3210	Late Bloom	Second	Sun	.50	5	4.6	.537	.2884	1	3.2	.081	.0066	1

Table 40.—Statistical Examination of Table 20 and Seasonal Progression—Vitamins B and G.

Sample No.	Stage of Growth	Cutting	Level Fed in g.	No. of Rats	VITAMIN B: Vit. B in 1 g. Hay	VITAMIN B: Probable Error	VITAMIN B: (P.E.)²	VITAMIN B: Rank	VITAMIN G (all fed .35 g. hay): Vit. B in 1 g. Hay	VITAMIN G: Probable Error	VITAMIN G: (P.E.)²	VITAMIN G: Rank
3192	Early Bud	First	.50	5	6.0	.335	.1122	1	4.3	.196	.0380	1
3203	Early Bud	Second	.50	5	5.5	.302	.0912	1	4.3	.207	.0428	1
3213	Early Bud	Third	.50	5	4.1	.314	.0986	2	4.0	.154	.0237	1
3193	Early Bloom	First	.50	5	5.0	.407	.1656	1	5.3	.407	.1656	1
3202	Early Bloom	Second	.75	5	4.1	.130	.0169	2	3.4	.251	.0630	3
3220	Early Bloom	Third	.50	5	2.6	.531	.2820	3	4.2	.404	.1632	2

Table 41.—Statistical Examination of Table 21—All Samples—Third Cutting, Early Bloom—Effect of Mold on Vitamin B.

Sample No.	Level Fed in g.	No. of Rats	Vit. B in 1 g. Hay	Probable Error	(P.E.)²	Rank	Treatment
3230	.50	5	6.7	.105	.0110	1	No rain
3231	.50	5	5.2	.152	.0231	2	1.5" rain in Swath
3233	.50	5	4.6	.329	.1082	2	2" rain in Cock
3232	.50	5	4.4	.371	.1376	2	1" rain in Cock
3212	.50	5	3.1	.181	.0328	3	1" rain in Swath
3211	.75	5	1.0	.151	.0228	4	1" rain in Cock
3227	.75	5	0.5	.176	.0310	4	2" rain in Cock (Rain intermittent)

Table 42.—Statistical Examination of Table 29—Vitamin G in Hays from Other States.

Sample No.	Origin	Stage of Growth	Cutting	Level Fed in g.	No. of Rats	Vit. G in 1 g. Hay	Probable Error	(P.E.)²	Rank
3241	Southern B.	Dehydrated		.35	5	2.9	.132	.0174	1
3238	Southern A.	Early Bloom	Third	.35	5	2.7	.105	.0110	1
3242	Southern B.	Field Cured		.35	5	2.4	.340	.1156	2
3236	Southern A.	Early Bloom	Second	.35	5	1.5	.086	.0074	3
3235	Eastern	Early Bloom	Second	.35	5	1.3	.311	.0967	4
3237	Southern A.	Full Bloom	Third	.35	5	0.8	.096	.0094	5
3234	Eastern	Early Bloom	First	.35	5	0.6	.212	.0449	6

TABULAR SUMMATION

For a general summation of the results of this bulletin several brief tables are added so that the vitamins can be compared more readily. The results are given in units per gram of hay.

Effect of Maturity of Plant on Vitamin Content Cured in Diffused Light.

	VITAMIN A			VITAMIN B			VITAMIN G		
	Early Bud	Early Bloom	Late Bloom	Early Bud	Early Bloom	Late Bloom	Early Bud	Early Bloom	Late Bloom
First Cutting	40	60	....	6.0	5.0	....	4.3	5.3	....
Second Cutting	16	32	9	5.5	4.1	7.0	4.3	3.5	2.3
Third Cutting	24	64	....	4.1	2.6	....	4.0	4.2	....

Loss of Vitamins by Rain—Sun-cured.

VITAMIN A

	No rain	1 inch rain	Percentage loss	2 inch rain	Percentage loss
Early bloom first cutting	36	16	55.6	9.5	73.6
Early bloom second cutting	18	20	....	13.0	27.8
Early bloom third cutting	28	12.5	55.4	12.5	55.4
Early bloom third cutting (in cock)	28	....	....	22	21.4
Early bud third cutting	42	32	23.8	16	61.9

Loss of Vitamins by Rain—Sun-cured.—Continued

	VITAMIN B			VITAMIN G		
	No rain	1 inch rain	2 inch rain	No rain	1 inch rain	2 inch rain
Early bloom first cutting	2.1	5.7	3.5	4.9	3.6	3.8
Early bloom, second cutting	2.6	3.2	3.8	3.5	3.7	4.4
Early bloom third cutting	1.7	1.3	1.9	3.7	4.2	3.9
Early bloom third cutting (in cock)	1.7	.5	1.6	3.7	....	3.7
Early bud third cutting	2.5	4.2	1.6	3.6	4.5	4.7

Loss of Vitamins by Curing in Sunlight.

	VITAMIN A		VITAMIN B		VITAMIN G	
	Diffused Light	Sunshine	Diffused Light	Sunshine	Diffused Light	Sunshine
Early bloom first cutting	60	36	5.0	2.1	5.3	5.0
Early bloom second cutting	32	18	4.1	2.6	3.5	3.5
Early bloom third cutting	64	28	2.6	1.7	4.4	3.7
Late bloom second cutting	9	16	7.0	4.6	2.3	3.2
Early bud third cutting	24	42	4.1	2.5	4.0	3.6

Effect of Molds.

Season 1931

Third cutting	VITAMIN A	VITAMIN B	VITAMIN G
Check (diffused light)	76	6.7	3.3
Moldy (bell jar)	32	......	......
Moldy (1½ inches rain—swath)	18	4.5	3.0
Moldy (1 inch rain—cock)	40	4.4	2.9
Moldy (2 inches rain—cock)	38	4.6	1.9

Vitamins in Alfalfa Hays from Other States.

	VITAMIN A	VITAMIN B	VITAMIN G
East—first cutting	5.0	4.5	.6
East—second cutting	24.0	5.4	1.9
South A	9.0	6.0	1.5
South A	2.5	4.1	.4
South A	18.0	5.3	2.7
South—field cured B	24.0	4.3	2.4
South—dehydrated B	60.0	7.0	3.0
Colorado, third cutting, 1931	76.0	6.7	3.3

LITERATURE CITED

(1) Abbott, O. D.
1927 Florida Station Report. pp. 89, 90;
1929 Florida Station Report. pp. 59, 60;
1931 Florida Station Report. p. 81.

(2) Bethke, R. M., and Kick, C. H.
1929 Ohio Station Bul. 431. p. 117.

(3) Cady, O. H., and Luck, J. M.
1930 Jour. Biol. Chem. 86:743.

(4) Davis, D. E., and Beach, J. R.
1925 California Station Bul. 384.

(5) Dutcher, R. A., and Wilkins, S. D.
1921 Amer. Jour. Physiol. 57:437.

(6) Eddy, W. H., Heft, H. L., Stevenson, H. C., and Johnson, R.
1921 Jour. Biol. Chem. 47:249.

(7) Eddy, W. H., and Stevenson, H. C.
1920 Jour. Biol. Chem. 43:295.

(8) Hart, E. B., McCollum, E. V., Steenbock, H., and Humphrey, G. C.
1917 Jour. Agr. Research. 10:175.

(9) Hathaway, J. L., Davis, H. P., and Graves, R. R.
1932 Nebraska Station Research Bul. 62.

(10) Hauge, S. M., and Aitkenhead, W.
1930 Indiana Station Report. p. 74;
1931 Jour. Biol. Chem. 93:657.

(11) Headden, W. P.
1896 Colorado Station Bul. 35.

(12) Helyar, F. G.
1927 New Jersey Station Report. p. 73.

(13) McCollum, E. V.
1917 The Harvey Lectures. 12:151.

(14) McCollum, E. V., and Simmonds, N.
1917 Jour. Biol. Chem. 32:347.

(15) McCollum, E. V., Simmonds, N., and Pitz, W.
1916 (a) Amer. Jour. Physiol. 41:361;
1917 (b) Jour. Biol. Chem. 30:13.

(16) O'Brine, D.
1889 Colorado Station Bul. 8.

(17) Osborne, T. B., and Mendel, L. B.
1919 (a) Jour. Biol. Chem. 37:187.
1919 (b) Jour. Biol. Chem. 39:29.
1920 (c) Jour. Biol. Chem. 41:549.

(18) Russell, W. C.
1927 New Jersey Station Report. p. 58.
1929 Jour. Biol. Chem. 85:289.

(19) Sherman, H. C., and Smith, S. L.
1931 The Vitamins (book).

(20) Shuey, R. C.
1914 Jour. Ind. Eng. Chem. 6:910.

(21) Silva, S. S., Golding, J., Drummond, J. C., and Coward, K. H.
1921 Biochem. Jour. 15:427.

(22) Smith, M. C., and Lynott, M. L.
1931 Jour. Agr. Research. 42:421.

(23) Steenbock, H., and Boutwell, P. W.
1920 Jour. Biol. Chem. 41:163; 42:131.

(24) Steenbock, H., and Gross, E. G.
1920 Jour. Biol. Chem. 41:149.

(25) Steenbock, H., Nelson, M. T., and Black, A.
1924 Jour. Biol. Chem. 59:IX; 62:275.

(26) ——————
1931 (a) Indiana Station Report;
1925 (b) Kansas Station Tech. Bul. 15;
1931 (c) Nebraska Station Report;
1929 (d) New Jersey Station Report, p. 8.