THE USE OF THE PROPHYLACTIC METHOD FOR THE DETERMINATION OF THE VITAMIN A CONTENT OF FEEDS

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THESIS

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INTRODUCTION and HISTORY

In recent years some manufacturers of patented foods,such as dog foods and other cereal products, have experienced a great deal of difficulty in placing on the market a food which they could guarantee to contain a definite vitamin A potency.This difficulty was not so much in finding a source of vitamin A as it was in preventing the loss of the vitamin during the storage of the food.

Cod liver oil has been known to be a very good source of this vitamin,and as such has been added to foods to fortify the vitamin A potency.When kept in the cold at $0^{o}C$,cod liver oil is quite stable so far as some of its constituents are concerned,but the vitamin A potency of cod liver oil kept at $0^{o}C$ has been found to decrease even at this low temperature. The length of time the vitamin A potency of cod liver oil will be retained has been found by Fraps and Kemmerer (1) to vary with the temperature and the food with which it is mixed.At higher temperatures the stability of the vitamin is greatly decreased.

The vitamin A activity of yellow and green colored plant materials has been traced to the presence of carotenoid pigments alpha,beta and gamma carotene by von Euler,Karrer,Hellstrom and Rydbom (2),and more recently also to xanthophyll pigment,kryptoxanthin,

by Kuhn and Grundmann (3).These "precursors" of vitamin A have also been used as supplements in foods and they too have been found to be unstable.Fraps and Kemmerer (1) dissolved them in oil which was then mixed with various feeds.The mixtures were stored under various conditions and examined periodically. It was found that practically all of the vitamin A value was lost after four weeks of storage.When hydroquinone was used as a stabilizer the vitamin A potency did not decrease as quickly,but even then most of it was lost after three weeks of storage.Fraps and Kemmerer (1) believe that the addition of fish oils or their concentrates to feed mixtures,for the purpose of increasing the vitamin A potency,is of little value unless the feed is used immediately. They found that from 79% to 100% of the vitamin A disappeared after four weeks of storage at either 7°C or 28°C.Feeds stored in large amounts lost their vitamin A from fish oils as rapidly as feeds stored in small amounts.Fraps and Kemmerer (1) found also that carotene dissolved in vegetable oil after being added to feeds was more stable than the vitamin A in cod liver oil,especially when the mixture was stored at low temperatures.

Carotene in alfalfa and kryptoxanthin in yellow corn were also found to be unstable,although they

were not destroyed as rapidly as carotene dissolved in oil.Carotene in alfalfa meal was found to be more stable at 6°C than at room temperature.Alfalfa stored at a temperature of 35°C lost carotene rapidly at first and then quite slowly,indicating that some of the carotene was easily destroyed,while a portion was apparently protected so that it was less readily destroyed.Heywang and Titus (4) found that alfalfa meals varied considerably in their vitamin A potency and that it was unwise to rely on a supplement of less than 5% of any type of meal unless its vitamin A potency was known.The variability of commercial meals in this factor is due in part to the length of time the alfalfa is exposed to the sunlight. Krizenecky (5) has expressed the opinion that the vitamin A value is preserved best by artifical drying and that the greatest losses occur when hay is dried as usual in full sunlight.Russell (6) has done considerable work on the effect of drying of alfalfa on the vitamin A value and his results substantiate those of the previous authors.He also found that yellow dent corn was about 50% more potent with reference to its vitamin A value than a white capped dent variety. Esselen,Fellers and Isgur (7) found that the kernels of yellow maize lost much of their vitamin A potency as they matured and dried out.

Fraps and Treichler (8) in studying the effect of storage on the vitamin A value of dried foods observed a gradual loss in the case of alfalfa leaf meal,yellow dent corn and powdered whole milk.Grinding corn before storage did not seem to increase to any noticeable extent the loss of the vitamin A potency in the yellow corn meal as compared with the whole grain.

Kon (9) tested certain patented dog foods on rats and found that they differed considerably in nutritive value.Even the best of them did not produce the rate of growth obtained with the stock ration used in his laboratory.Most of these dog foods appeared to be deficient particularly in vitamin A.The effects of a continued diet deficient in vitamin A are to be noted in experimental xerophthalmia. Steenbock,Nelson and Hart (10) produced this condition in dogs after ninety four days.A knowledge of the vitamin A value of dog foods and the effect of storage are therefore important.Because of the physiological importance of vitamin A and its instability,the assay of foods and food mixtures is obviously necessary.

The curative method for the biological assay of foods for their vitamin A value is at present the official method.Rats weighing from 40 to 50 grams

and less than twenty eight days old are fed a vitamin A-free diet for a period of 24 to 45 days in order to deplete them of their vitamin A reserves.The point of depletion is indicated by the failure of the rats to gain in weight.At this stage the rats are fed the test material and the reference oil daily.The response of the rats of the assay and the reference groups is calculated on the basis of the difference between the average weight of the surviving rats and the average weight of the same rats on the day beginning the assay period.Rats that gain between 12 and 60 gm. and which consume the prescribed dose of oil or test material for at least 22 days of the assay period are considered valid.The units of vitamin A per gram of test material are determined by dividing the daily dose in milligrams of the reference oil necessary to produce in a reference group an average gain in weight,"G",of not less than 12 gm. and not more than 60 gm.,by the daily dose in milligrams of the test material that will produce in an assay group an average gain in weight equal to or greater than "G",and multiplying the result of this division by the units per gram of vitamin A contained in the reference oil.

Sherman and Todhunter (11) gave single feedings of carotene,of cod liver oil,of kale or of calf liver to standardized rats depleted of their surplus body

stores of vitamin A.The weight curves throughout the period of survival after such feedings were then charted on a fixed scale and the areas bounded by the resulting curves were measured with a planimeter.Within limits amply sufficient for experimental work,increasing the amount of the vitamin A or of the "precursor" given in a single feeding gave an approximately proportional increase in the area under the curve. Carotene curves were used as a standard of reference. Direct comparison showed that the method of single feedings results in data which can be evaluated in terms of the same units and with findings in close agreement with those of the methods of feeding through periods of four or more weeks as hitherto regarded most accurate and conclusive.The single feeding method eliminates any possibility of loss of vitamin A in the material when it is stored.Storage is necessary when methods requiring subsequent feedings are employed. Besides eliminating the storage of material the method has another advantage in that it saves considerable time.

In this work an attempt was made to determine whether or not a prophylactic method could be used advantageously,that is,shorten the time period without sacrificing accuracy.Richards and Simpson (13) are of the opinion that the persistence of pathological

conditions that have been induced by vitamin A deficiency,taken in conjunction with their diverse nature, forms the strongest argument against the curative method of assay.In the curative test,vitamin A can influence growth only indirectly by effecting improvement in the pathological conditions,and it is quite obvious that no consistency in results can be expected when these conditions vary so widely not only in their nature but also in their intensity and curability.It has been shown that with a comparatively short depletion period there is more likelihood of a good response to vitamin A dosage,but even a considerable reduction in the length of this period cannot insure similarity in the condition of the experimental animals.It was suggested that any biological assay of vitamin A to be satisfactory must be of the prophylactic type.Its object should be to determine not the amount of vitamin A which will produce fairly satisfactory weight curves in animals of inferior type,but the amount which will keep normal animals in a normal condition as evidenced by the weight curves and also by examination of the tissues after death.

Accordingly,the object of this study was to determine whether a prophylactic method could be used for the vitamin A assay of a variety of feeds.

EXPERIMENTAL

In carrying out these assays with albino rats, care was taken in establishing the groups of test animals so that all the groups were comparable.That is,whenever an animal was assigned to one group,a comparable animal of the same sex,age,litter,weight and dietary history was assigned to each other group. Each group was composed of two males and two females. Since the stock diet was relatively rich in vitamin A it is certain that the animals used in this study had a reserve of vitamin A at the beginning of the experimental period.This reserve was undoubtedly acquired in the following three ways: a) by passage through the placenta to the foetal circulation; b) by ingestion post-natally by suckling; c) by direct consumption of the stock ration.Due to this storage of the vitamin A it seemed advantageous to remove the young rats from their maternal environment just as soon as they became old enough to be used for experimental purposes.It was determined that animals weighing from forty to fifty grams and from twenty to twenty two days old were most suitable for the assay,and animals of that weight and age were used throughout this work.

The rats were kept in individual cages during the entire experiment.The cages,made of wire mesh,were

about ten inches in diameter and ten inches in height. The bottoms of the cages were raised and the mesh was large enough to allow the feces and the urine to pass through and be collected in a pan.A metal feeding cup was used for the vitamin A-free diet.

Instead of depleting the rats of their vitamin A store and then supplementing the basal diet with the test material,as is the procedure in the curative method of assay,the rats were fed the vitamin A-free diet "ad libitum",and had access to all the water they cared to drink during the experimental period of six weeks.Once each week the rats received in addition

Vitamin A-Free Diet

Dextrin	67.0%
Casein (Vitamin-Free)	18.0%
Crisco	5.0%
Dried Brewer's Yeast	5.0%
" " " (Irradiated)	1.0%
Salt mixture (Steenbock's 40B)	4.0%

to the above diet a definite amount of the test material.When it was found that the supplement was not relished by the rats it was mixed with the basal ration and then fed.The approximate vitamin A potency of the foods was known before the assays were begun, thus eliminating much preliminary experimentation.

Six feeds were assayed,three being mixed dog foods and the others,alfalfa leaf meal,yellow corn meal and dried whole milk powder.

The Gro-Pup dog food was indicated to contain 1560 International (U.S.P.) units of vitamin A per pound of food.A representative sample was taken and fed at the following two levels as the supplement, 4.45 grams to one group of rats and 8.90 grams to another.These amounts of food were expected to contain a vitamin A potency of 15 and 30 units (U.S.P.) respectively.Each rat in one group received 4.45 grams of the food once a week,while each rat of the second group received 8.90 grams of the food weekly.The Fox Cube and the Vita Ration # 1 dog foods were expected to contain 3400 units (U.S.P.) of vitamin A per pound.On this basis two grams of either of these dog foods should contain 15 units (U.S.P.) of vitamin A and four grams 30 units (U.S.P.) of vitamin A. These quantities of the two dog foods were fed to four other groups of rats.

The vitamin A potency of alfalfa varies considerably.Fraps,Copeland and Treichler (13) found a sample to contain about 32.5 units (U.S.P.) per gram.On the basis of their results it was decided that the alfalfa be fed at three levels,that is 0.25 gram,0.50 gram and 1.0 gram weekly.In the case of the yellow

corn meal and the whole milk powder there was also considerable uncertainity as to the probable vitamin A potency.Fraps and Treichler (14) found the vitamin A content of corn meal to vary from 4 to 7 units (U.S.P.) per gram.Oonsequently it was decided to feed the corn meal at three levels,1.0 gram,2.0 grams and 5.0 grams. Rice and Munsell (15) found that dried whole milk powder contained from 8 to 28 units (U.S.P.) of vitamin A per gram,while Fraps and Treichler (14) found a sample to contain from 9 to 14 units (U.S.P.) per gram.With this food it was also decided to feed supplements of 1.0 gram,2.0 grams and 5.0 grams weekly.

For comparison there was used a standard reference oil distributed by the Board of Trustees of the United States Pharmacopoeial Convention.This oil contained 3000 units (U.S.P.) of vitamin A per gram of oil and was diluted with Wesson oil in such a proportion that 1 cc. of the diluted standard oil contained 30 units (U.S.P.) of vitamin A.A few crystals of hydroquinone were added as a preservative and the oil was stored in a refrigerator at about 0°C.this oil was fed at levels of 10,15,20 and 30 units weekly to four groups of rats.A group receiving no supplement was included in this series.

All of the supplements were fed in separate, flanged dishes so as to minimize wasting of the feed.

The dishes were left in the cages until all of the supplements were eaten.The rats were weighed once each week.The gains in weight of each group were averaged and appear as the average total gain in Table # 1.The total gains of the controls over the six week period were plotted against the number of units of vitamin A in the supplement.Then by referring the average total gains of the groups of rats receiving the feed supplements to this curve,the units of vitamin A of each supplement could be determined quite accurately.By dividing the number of units by the number of grams fed,it was possible to determine the number of units of vitamin A per gram of the feed.The results obtained from each level were then averaged and they appear in Table # 2.

Table # 1

Controls

Supplement	Units of Vitamin A	Total Gain
0.00 cc. of St'd oil	0	55 grams
0.33 " " " "	10	102 "
0.50 " " " "	15	116 "
0.66 " " " "	20	125 "
1.00 " " " "	30	141 "

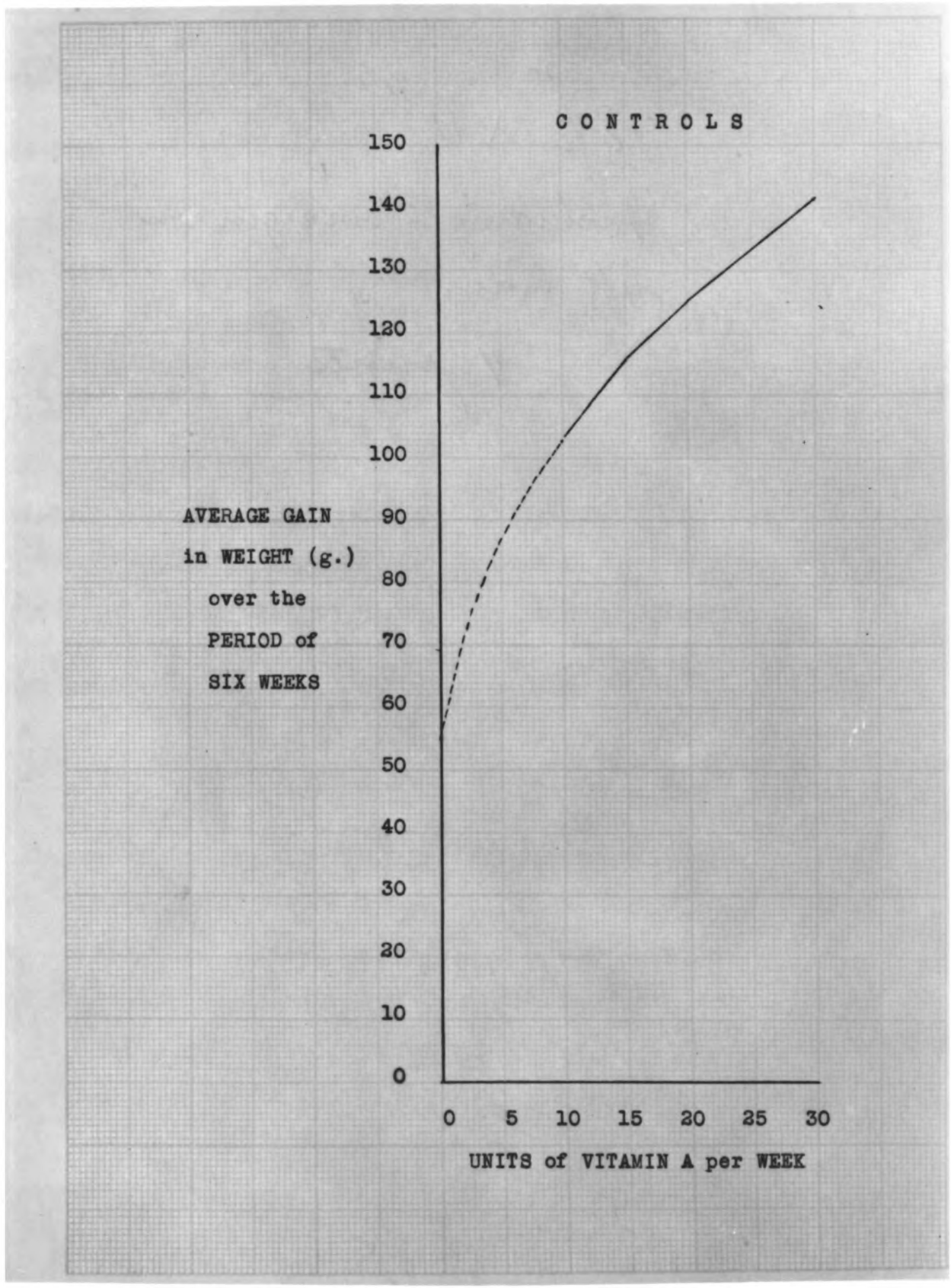
CONTROLS
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
AVERAGE GAIN
in WEIGHT (g.)
over the
PERIOD of
SIX WEEKS
0
5
10
15
20
25
30
UNITS of VITAMIN A per WEEK

Table # 2

Feeds

Feed	Total Gain	Supp't	Units/g. (U.S.P.)	Average Units/g.
Gro-Pup	77g.	4.45g.	0.67	
" "	93g.	8.90g.	0.73	0.7
Fox Cube	108g.	2.00g.	6.0	
" "	121g.	4.00g.	4.4	5.2
Vita Ration # 1	71g.	2.00g.	1.0	
" " " "	107g.	4.00g.	2.9	1.95
Alfalfa	100g.	0.25g.	36.0	
"	124g.	0.50g.	38.0	
"	141g.	1.00g.	30.0	34.6
Corn meal	102g.	1.00g.	9.8	
" "	136g.	2.00g.	13.1	
" "	141g.	5.00g.	6.0	9.6
Milk powder	140g.	1.00g.	29.25	
" "	176g.	2.00g.	-- --	
" "	174g.	5.00g.	-- --	29.25

DISCUSSION of RESULTS

The curve obtained by plotting the gains in weight of the rats receiving the different levels of the official reference oil was very smooth and therefore appeared to be suitable as a standard for comparison in the evaluation of the vitamin A potency of various food stuffs.

The milk powder used in this experiment had a much higher vitamin A content than expected.Consequently the amounts of the milk powder used as supplements were too large for a comparison of the growth data with those of the reference oil groups.The growth obtained with the two and five gram levels was approximately the same,indicating that 2 grams of milk powder contained sufficient vitamin A to give maximum growth when fed in conjunction with the basal ration.The levels for the corn meal and the alfalfa on the other hand were very satisfactory because the growth responses fell within the range of those given by the reference oil.

Fraps,Copeland and Treichler (13) found alfalfa to contain 32.5 units (U.S.P.) per gram,while by the prophylactic method used in this study the content was found to be 34.6 units (U.S.P.) per gram.Fraps and Treichler (14) found the vitamin A potency of corn meals to vary from 4 to 7 units per gram.By the

prophylactic method an average value of 9.6 was obtained.In the case of the milk powder,of which only one level of the supplement could be used for comparison,a potency of 29 units per gram was obtained by the prophylactic method,whereas Rice and Munsell (15) found that samples contained from 8 to 28 units per gram,and Fraps and Treichler (14),9 to 14 units per gram.As a result of the fairly close agreement of the results,it is reasonable to believe that the prophylactic method of assay is dependable for the determination of vitamin A in these types of feeds.

From the results it is evident that the vitamin A content of the dog foods was not as high as anticipated.This was shown also by a comparative feeding test in which alfalfa was used to increase the vitamin A supply.Although it is not definitely known how long the dog foods had been stored before they were used in this assay,it is certain that storage decreased the vitamin A potency because known amounts of vitamin A (Cod liver oil) had been added when the food was prepared.The Vita Ration # 1 was prepared in the form of a meal while the Fox Cube was prepared in the form of compressed cubes about ½ inch in diameter and slightly more than ½ inch in length.Both foods originally had the same vitamin A potency,yet upon biological assay after a few months of storage it was found that

the exclusion of air by preparing the food in the cube form apparently greatly decreased the loss of the vitamin A potency.Similar conclusions on different foods were made by Taylor and Russell (16) and also by Fraps and Kemmerer (1),who found that large compact samples of feeds lost their carotene at a less rapid rate than small samples loosely packed.

The units of vitamin A per gram of Gro-Pup,Vita Ration # 1 and Fox Cube were found to be .7,1.95 and 5.2 respectively.In comparative feeding tests carried out previously,each of the dog foods was supplemented with alfalfa,an excellent source of "pro-vitamin A", and the growth responses were tabulated.After ten weeks it was noted that the supplement affected the greatest increase in growth response with the Gro-Pup, less with the Vita Ration # 1 and least with the Fox Cube.This substantiates the results obtained by the prophylactic method and indicates that the method in question may be used for the determination of the vitamin A potency of a wide variety of foods and food mixtures.

SUMMARY

1. The prophylactic method of assay, when used in conjunction with an official reference oil as a standard for comparison, gave results with alfalfa, corn meal and milk powder which are in close agreement with those obtained by the official curative method.

2. The prophylactic method was also applied with satisfactory results to the determination of the vitamin A potency of several complete dog food mixtures.

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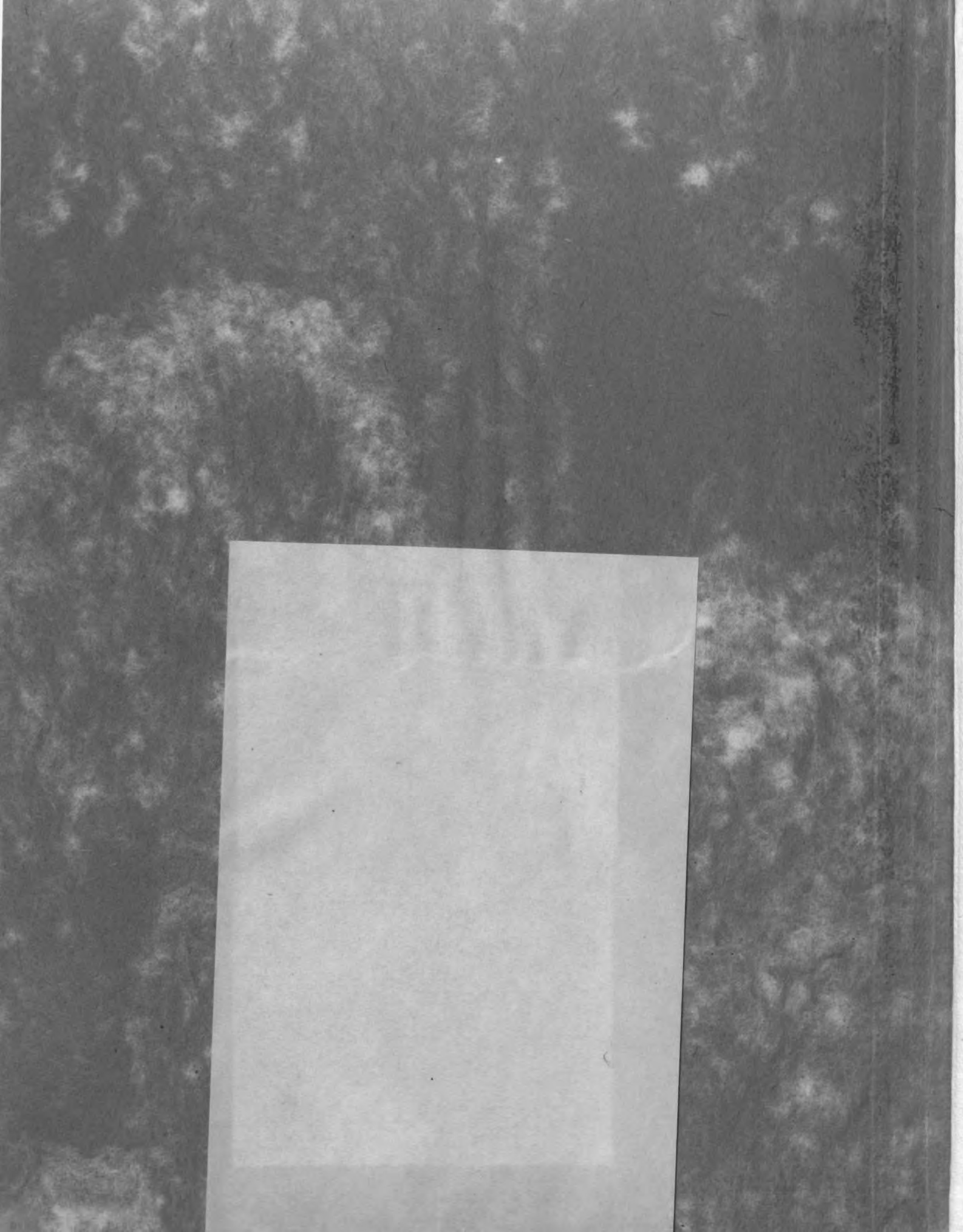

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