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Studies in vitamin A technic

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STUDIES IN VITAMIN A TECHNIC

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CONTENT

LIST OF TABLES AND CHARTS

LIST OF TABLES AND CHARTS (Cont.)

INTRODUCTION

In biological researches, especially those involving the use of experimental animals, the results obtained are often of such irregularity that the interpretation of the data is a perplexing part of the investigation. This is, in large measure, due to that element termed "individual variation" which is an admission of the fact that we are as yet unable to control many of the factors affecting our results. The biologist does not have at his command the precision of technic employed by the chemist or the physicist. It is therefore advisable in biological work to use statistical method as an aid in the interpretation of data in order to eliminate, in so far as possible, unwarranted conclusions due to chance variations. Statistical method can also be used as a tool in determining the general trend of reactions now on record with a view to making predictions as to future reactions. Such predictions must, of course, be verified by experimental data.

The methods of vitamin assay are based upon the theory that when all food essentials except one vitamin are present in the diet in adequate amounts, the gain in weight is due to the vitamin added as a supplement. Even when performed under carefully standardized technic the gains in weight made by the experimental animals show considerable variability. Because of this lack of uniformity it was thought advisable to make a statistical study of the data now on record in this laboratory with a view to determining, if possible, the causes of the variability in our animals and to devise changes in technic which might lead to more accurate results.

The work reported in this thesis is divided into four sections. Section I deals with the means and correlations of the variables measured during the experimental period of a vitamin A test, together with the per cent effect of these variables upon the gain in weight. The second section is a report of the influence of the composition of the basal diet on the responses of vitamin A test animals during the depletion period. The third section is an analysis of the reactions of vitamin A test animals from different stock colonies and the fourth a report of an attempt to reduce the variability of the weight gains by controlling the food intake.

EXPERIMENTAL

In the biological method for the assay of vitamin A, standard test animals are maintained upon a basal diet free of vitamin A until the body store of that vitamin is depleted. During this preliminary or depletion period the animals grow at approximately a normal rate until the body store of vitamin A wanes. The growth curves then flatten and the animals begin to show the symptoms characteristic of vitamin A deficiency. At a point at which experience indicates that the body store of vitamin A is exhausted measured portions of the supplementary vitamin containing food are added to the diet. The weight of the animal at this point is considered the initial weight of the test period and is identical with the final weight of the depletion period. The gain in weight made by the animal during the experimental period, i.e., the eight weeks following the depletion period, is considered a measure of the vitamin potency of the food under investigation.

The vitamin tests performed in this laboratory have had to do largely with the effect of cultural conditions and degree of maturity upon the vitamin content of vegetables. In performing these experiments a relatively large variation in the reactions of the test animals has been found. This is especially true in the case of vitamin A tests. In one experiment for instance, two standard test animals received exactly the same treatment during both the depletion and experimental periods.

Both were fed 0.5 grams of tomato as a source of vitamin A. One of these animals ♀ 3692 gained 5 grams during the 8 weeks experimental period and a litter-mate ♀ 3690 gained 57 grams. Both of these animals weighed 40 grams at 28 days of age and their weights at the end of the depletion period were 95 and 91 grams respectively. Such marked differences in gain make it necessary to use relatively large numbers of animals in order to discriminate between differences. Because of this variation in the responses of the animals we became interested in making a statistical study of their reactions in an attempt to discern reasons for this extreme variation.

The technic used in all of the vitamin work in this laboratory has been essentially that of Ferry (1) each rat being kept in an individual all metal cage with a false bottom. The basal diet and distilled water were fed ad libitum and the daily allotment of the vitamin containing food was fed separately. Daily food consumption records were kept and the animals weighed once each week or more often. The data obtained from these experiments were tabulated for this statistical study under the following headings: animal number, strain, sex, initial weight, final weight, total experimental gain, days on experiment, vitamin studied, weight of the vitamin containing food, kind of vitamin containing food, condition of vitamin containing food, total basal diet, kind of basal diet, and average daily consumption of the basal diet.

The first study made was a comparison of the means and correlations of the measured variables. The most significant correlation was found to be that between the quantity of basal diet ingested and the gain in weight, 0.83 ± 0.02. This correlation coefficient is relatively high and led to the belief that it might be possible to reduce the variability in the weight-gains by controlling the food intake.

Two vitamin A free diets have been used in the laboratory. One of these diets contained 22 per cent of Crisco* and the other contained no fat. The differences in the reactions of the test animals fed these diets indicated that the vitamin A free diet containing fat was preferable because it shortened the depletion period. This possibility was tested in a more carefully controlled experiment but no significant differences were found.

During the course of the work of this laboratory two stock colonies of rats have been used. A study was made of the differences in the reactions of vitamin A test animals from these two colonies. Significant differences were apparent.

The records of 123 vitamin A test animals were separated out for further study. These animals were all offspring of an inbred stock colony. Their parents had all been fed the same stock ration. The test animals had been treated exactly alike during the depletion period of a vitamin A test. It was thought that these data offered an opportunity to study the effect of food intake upon the gain in weight. A method of

*Crisco is a hydrogenated vegetable oil.

controlling the food intake which was based upon the knowledge gained by studying the reactions of these 123 test animals was devised. The method was then tested experimentally.

The results of the studies briefly outlined above are reported in detail under the heading "Results".

RESULTS

Section I

Means and Correlations of the Measured Variables Together with the Per cent Effect of these Variables on the Gain in Weight

The records of 429 vitamin A test-animals were tabulated as described above and a study was made of the reactions of the animals during the experimental period of a vitamin A test. For the purpose of this study it has been assumed that the animals received the same quantity of vitamin, which is not strictly true, as they have been fed different kinds and different amounts of vegetables as the source of the vitamin under investigation. It may be stated here that in all our vitamin work an attempt has been made to approach the three gram gain per week for vitamin A tests, so that the data are not as heterogeneous as might be supposed at first thought.

The animals used in these tests were albino rats twenty-eight days of age. It has been shown by several investigators that the diet of the mother rat during pregnancy and lactation is a determining factor in the growth response of the young rats used for vitamin tests. The rats whose records were used in this study were not all reared on the same basal diet. Approximately seventy-five per cent of the animals were reared in this laboratory, where it is standard practice to reduce all litters to eight rats. The mothers of these animals received a modified form of Steenbock's basal ration which has the following composition: yellow corn meal 64, linseed oil meal 16, yeast 2, wheat germ 10, crude casein 5, ground alfalfa 2, NaCl .5, and $CaCO_3$ .5. Ten cc. fresh whole milk was fed daily. The other twenty-five per cent of the animals were supplied by our Chemistry Department. Their mothers were fed a diet of the following composition: oat groats 35, yellow corn 38, alfalfa 5, wheat 9, tankage 6, linseed oil meal 5, and dried buttermilk 3. During the course of the experimental work, two basal diets have been used, namely, those of Sherman and Munsell (2) and of Osborne and Mendel (3). The composition of each of these rations with their approximate caloric value per gram of the basal diet is given in Table I. The chief difference between the two vitamin A free diets is the amount of fat they contain.

TABLE I

Composition of the Two Basal Diets

Vitamin A Free Diets

	Sherman & Munsell Vitamin A Free Diet	Osborne & Mendel Vitamin A Free Diet
Vitamin A free casein	20	18
Starch	70	51
Yeast	5	5
Salts (6)	4	4
Crisco	-	22
NaCl	1	-
Calories per gram of diet	3.6	4.7

Comparisons of Means and Coefficients of Variability

The data of the experiments were separated into two groups according to the basal diet fed, thus making two groups. The means were calculated in order to detect any striking differences between the two groups of animals. The means, standard deviations, and coefficients of variability are shown in Table II.

TABLE II

Mean Initial and Final Weights, Standard Deviations and Coefficients of Variability of Rats Fed Different Basal Diets Free of Vitamin A

	Sherman & Munsell Vitamin A Free Diet	Osborne & Mendel Vitamin A Free Diet
Number of rats	120	349
Mean initial weight	110 ± 2.0 Gms.	112 ± 1.1 Gms.
Standard deviation of initial weight	32 Gms.	31 Gms.
Coefficient of variability of initial weight	29 %	28 %
Mean number of days on depletion	46 ± 0.7 days	40 ± 0.3 days
Standard deviation of days on depletion	11 days	8.9 days
Mean final weight	161 ± 2.8 Gms.	144 ± 2.0 Gms.
Standard deviation of final weight	46 Gms.	55 Gms.
Coefficient of variability of final weight	28 %	38 %
Mean gain	51 ± 2.5 Gms.	32 ± 1.9 Gms.
Standard deviation of gains in weight	40 Gms.	53 Gms.
Mean calories eaten during the experimental period	1902	1458
Grams gained per gram of food ingested	0.097	0.104
Grams gained per calorie ingested	0.027	0.022

It will be seen in Table II that the mean initial weights with their standard deviations and coefficients of variability are practically the same for both diets. The average number of days required for depletion is less on the Osborne and Mendel diet containing fat than on the Sherman and Munsell fat free diet, the ratio of the mean difference to its standard deviation being 5.4. By examining the table further, it will be seen that during the experimental period the gain in weight on the Sherman and Munsell fat free diet is greater than on the Osborne and Mendel diet containing fat. In this case the ratio of the mean difference to its standard deviation is 4.3. The efficiency of the two diets calculated as grams gained per gram of diet consumed and the grams gained per calorie of diet consumed can also be seen from the table. Whether the difference in gain is due to the difference in caloric intake or to some metabolic property of the fat itself is a question. It will also be observed that whereas the initial weights and their standard deviations are practically the same on both diets, the standard deviation of the final weights of the animals fed the Osborne and Mendel basal diet is greater than that of the animals fed the Sherman and Munsell basal diet. The ratio of the mean difference to its standard deviation in this case is 4.7. What is the cause of this variability? Were these animals using their vitamin A for fat metabolism and consequently receiving less for protection against infections? Does this susceptibility to infection account for the greater variability in their reactions? If the animals making smaller gains are more variable in their reactions is a minimum gain of three grams per week a desirable standard? These questions must remain unanswered for the present.

Further Comparisons on the Basis of Gross Correlations

Correlations were run between the variables; initial weight, final weight, days on experiment, total food intake and total gain.

The more striking of these correlations are those between initial weight and total food intake, initial weight and total gain, total food intake and total gain. These correlations with their probable errors are recorded in Table III.

The biological significance of the correlations between initial weight and total food intake and between initial weight and total gain is questionable but the correlation between total food intake and total gain is undoubtedly significant.

Multiple Regression Coefficients and Regression Equations

For each group of data a multiple correlation coefficient (R) and a regression equation were calculated according to the method of Wallace and Snedecor (4). The independent variables were initial weight (A), days on the experiment (C), and total basal diet (D), and the dependent variable was total gain ($\bar{X}$). The coefficient and equation for the data of the vitamin A

TABLE III

Correlation Coefficients and Their Probable Errors

Vitamin A

Variables	Sherman & Munsell Vitamin A Free Diet	Osborne & Mendel Vitamin A Free Diet
	Correlation Coefficients based on 120 animals	Correlation Coefficients based on 349 animals
Initial weight and total food intake	0.31 ± 0.06	0.13 ± 0.03
Initial weight and total gain	-0.20 ± 0.06	0.20 ± 0.03
Total food intake and total gain	0.73 ± 0.03	0.83 ± 0.02

tests wherein the animals received the Sherman and Munsell vitamin A free diet are as follows:

Vitamin A. Sherman and Munsell vitamin A free diet.

$$R = 0.86$$

$$\bar{X} = 23 - 0.61\ A - 0.32\ C + 0.20\ D$$

This four variable regression equation is interpreted in the following manner:

The gain $\bar{X}$, increases:

(1) 0.61 grams per gram decrease in initial weight.

(2) 0.32 grams per day decrease in the experimental period,

and (3) 0.20 grams per gram increase in the total basal diet.

The similar multiple regression coefficient and regression equation for the group of animals receiving the Osborne and Mendel vitamin A free diet are as follows:

Vitamin A. Osborne and Mendel vitamin A free diet.

$$R = 0.91$$

$$\bar{X} = 43 - 0.58A - 1.96C + 0.43D$$

If we knew and could measure all of the factors affecting the gain in weight of an experimental animal and if all the relationships were linear, each multiple correlation coefficient would be 1.00. The difference between 1.00 and the square of the multiple correlation coefficient of any one group represents roughly the extent to which other uncontrolled and unmeasured factors, and curvilinearity affect the gain in weight. These multiple correlation coefficients are relatively high and represent our success in estimating the gain in weight from the three independent variables. In other words, they represent the extent to which the variables, initial weight, days on experiment, and total intake of basal diet influence the gain in weight. Score cards representing the per cent of the measured effect of each of the three independent variables on the dependent variable have been made according to the method of Wallace and Snedecor (4) and are given in Table IV.

It can be seen from Table IV that the amount of food ingested is by far the most important of these factors. In both groups the per cent effect of the amount of basal diet eaten is consistently greater than that of the initial weight or days on the experiment.

Sherman (5) has suggested that the initial weights of vitamin A test animals be controlled by controlling the diet of the mother and by establishing certain arbitrary limits of weight for the experimental animals in any one laboratory. Unless the animal dies before the expiration of the experimental period the time limit proposed is 56 days. Since these two factors have much less influence on the gain in weight than the amount of basal diet eaten would it not be well to consider some regulation of this factor?

TABLE IV

Score Cards Showing the Per Cent of the Measured Effect of Initial Weight, Days on the Experiment and Total Basal Diet on the Total Gain

	Initial Weight	Days on the Experiment	Total Basal Diet	Sum.
	Vitamin A. Sherman & Munsell Vitamin A Free Diet			
Partial Regression Coefficients	0.48	0.10	0.95	1.53
Scores or Rate Per Cents	31.4	6.5	62.1	100.0
	Vitamin A. Osborne & Mendel Vitamin A Free Diet			
Partial Regression Coefficients	0.35	0.73	1.57	2.65
Scores or Rate Per Cents	13.2	27.5	59.3	100.0

Section II

A Comparison of the Reactions of Vitamin A Test-Animals Fed Different Basal Diets during the Depletion Period

In the discussion of Table II Section I the question of the relationship of the fat content of the diet to the vitamin A requirement of the animal is raised. The data of Table II show a shorter depletion period for those animals receiving a diet containing fat. In vitamin A experiments a shorter depletion period is advantageous from the standpoint of economy of time, laboratory space and expenditure for food materials. It also places the animals on experiment at a lower age level and prevents the experimental period from extending beyond the logarithmic phase of the growth curve. If a higher per cent of fat in the diet will shorten the depletion period such a diet would be preferable for vitamin A tests.

Osborne and Mendel (6) have obtained good growth on diets low in fat. McAmis, Anderson and Mendel (7) state that rats will grow on diets free of fat but that better growth is obtained if a small percentage of fat is included in the diet. Whether the fat acts as a vehicle for the transport of vitamin A or whether the fat itself is responsible for the increment of growth is not determined. Liang and Wacker (8) are of the opinion that vitamin A is necessary for the synthesis of fat from glycerol and fatty acids. Nakaharo and Yokoyama (9) state that the absence of fat in the diet renders it possible for an animal to tolerate a vitamin A deficiency to some extent. In a paper dealing with the nutritive value of different fats Takahashi (10) states that vitamin A plays an important role in the combustion of fat in the animal tissue. He suggests that the body demands vitamin A in proportion to the amount of fat consumed and vitamin B in proportion to the amount of carbohydrate consumed. It was thought advisable to study the influence of the fat content of the basal diet on the reactions of the animals in this laboratory. Therefore two vitamin A free diets (Table I) were used over a period of several months with a view to comparing the reactions of the animals. One hundred and twenty three animals were fed the Osborne and Mendel diet and 60 the Sherman and Munsell diet. All of these animals were reared in this laboratory and were offspring of an inbred colony of stock rats. The technic used for the experimental animals was uniform throughout as was the technic of handling the stock colony. The latter were all fed upon the same stock ration. The mean initial weight, mean gain, mean days to depletion, and mean basal diet eaten daily for each of the two groups of experimental animals may be read in Table V.

The mean difference between the gains in weight is 4.4 and the standard deviation of this difference is 3.2. In the case of the days to depletion the mean difference is 3.0 and the standard deviation of the difference is 0.84. The difference between

TABLE V

Means and Probable Errors of the Variables Measured during the Depletion Period of Rats Fed Different Basal Diets Free of Vitamin A

	Osborne & Mendel Vitamin A Free Diet	Sherman & Munsell Vitamin A Free Diet
Mean initial weights, gms.	51.9 ± 0.29	55.3 ± 0.82
Mean gain during depletion, gms.	70.2 ± 0.87	74.6 ± 2.00
Mean days to depletion, days	36.0 ± 0.33	39.0 ± 0.46
Mean basal diet eaten daily, gms.	8.66 ± 0.06	10.67 ± 0.12
Mean calories eaten daily, calories	40.7	38.4

the weight-gains is not significant but that between the number of days to depletion is significant. These data bear out those presented in Table II and indicate that the vitamin A free diet containing fat is preferable to the fat free diet because it shortens the depletion period.

Section III

The Reactions of Vitamin A Test Animals from Different Stock Colonies

It was thought advisable to use for this study only the data of the depletion period of our vitamin A test animals as these data provide a larger number of cases wherein the animals were treated exactly alike. The data were tabulated under the following heads: rat number, sex, stock, initial weight, gain during depletion, days to depletion, and mean basal diet eaten daily.

When this laboratory was started a stock colony of albino rats was obtained from our chemistry department and was maintained without an organized scheme of mating until January 1928. At this time three pairs of the Wistar Institute inbred rats were obtained. These animals were from a strain that had been inbred for fifty generations. Continuing this strain by brother and sister matings we have developed a stock colony of inbred rats that has entirely replaced the former colony.

The entire group of records used in this study were divided into two groups, the Wistar stock and the Chemistry stock. The diet of the two stocks was identical. The vitamin A free basal diet fed to the entire group of experimental animals was that of Osborne and Mendel (3). Each group was then divided into males and females thus making four groups, Wistar males, Wistar females, Chemistry males and Chemistry females. The mean initial weight, mean gain, mean days to depletion, and mean basal diet eaten daily for each of these four groups of rats is recorded in Table VI. An examination of the differences in the reactions between the groups is recorded in Table VII.

By holding to the criterion of a mean difference being three times as large as its standard deviation as a test of significance and referring to Table VII, we see that there are no significant differences in the reactions of the males and females of the Chemistry stock. The only difference in the reactions of the males and females of the Wistar stock which is statistically significant is the difference in number of days required for depletion, the females requiring a longer period. Comparing the Chemistry stock with the Wistar stock we observe differences which are statistically significant between the mean initial weights and between the mean days required for depletion. The differences between the mean gains and the mean basal diet ingested by the two stocks of animals are not statistically significant.

It was thought that these differences warranted a separation of the two stocks but that the males and females of each stock might be grouped together for further study. The portion of a rat's life span covered by the depletion period preliminary to a vitamin A test is that from 28 to 70 days of age. At this period the animals have not matured sexually which probably accounts for the fact that there are no significant differences between the

TABLE VI

Means and Probable Errors of the Variables Measured during the Depletion Period of Animals Used for Vitamin A Tests

	Mean Initial Weight	Mean Gain	Mean Days to Depletion	Mean Basal Diet Eaten Daily
	gm.	gm.	days	gm.
Males from Chemistry stock	46.7 ± 0.46	71.0 ± 1.42	38.8 ± 0.40	7.96 ± 0.08
Females from Chemistry stock	44.5 ± 0.44	65.3 ± 1.40	40.2 ± 0.42	7.77 ± 0.08
Males from Wistar stock	52.7 ± 0.67	71.4 ± 1.19	34.4 ± 0.41	8.78 ± 0.09
Females from Wistar stock	51.2 ± 0.58	69.0 ± 1.26	37.5 ± 0.50	8.54 ± 0.08

TABLE VII

Mean Differences and Standard Deviations of the Differences between the Variables Measured during the Depletion Period of Animals Used for Vitamin A Tests

	Mean difference in initial weight		Mean difference in gain		Mean difference in days to depletion		Mean difference in basal diet ingested	
	M	σ	M	σ	M	σ	M	σ
	gm.	gm.	gm.	gm.	days	gm.	gm.	gm.
Males versus females of the Chemistry stock	2.2	0.9	5.7	2.9	1.4	0.9	0.19	0.5
Males versus females of the Wistar stock	1.5	1.3	2.4	2.5	3.1	1.0	0.24	0.6
Chemistry versus Wistar stock males	6.0	1.2	0.4	2.7	4.4	0.8	0.82	0.6
Chemistry versus Wistar stock females	6.7	1.1	3.7	1.9	2.7	1.0	0.77	0.5

sexes.

It was thought that a comparison of our data with those of another laboratory would be of interest. Similar data of vitamin A tests were very kindly furnished us by Dr. Hazel Munsell of the Bureau of Home Economics, U. S. D. A., Washington, D. C. in order that this comparison might be made. 120 vitamin A test animals of our laboratory stock that had been fed the Sherman and Munsell vitamin A free diet were used for this comparison. These animals are an entirely different group than any of those presented in Table VI. Dr. Munsell supplied data on 494 vitamin A test animals. The means and their probable errors together with the mean differences and their standard deviations for these two groups of rats may be read in Table VIII.

It will be seen that the differences in the reactions of the two stocks of animals are significant. It is evident that the results of our study could not be applied directly to the animals of Dr. Munsell's colony or to any other colony without first testing the homogeneity of the two groups of animals.

TABLE VIII

Comparison of the Measured Variables of Two Stocks of Rats Used for Vitamin A Tests. Bureau of Home Economics Data vs. Iowa State College Data

	Means		Mean Difference	
	Iowa State College n = 120	Bureau Home Economics n = 494	Difference	σ
Weight at 28 days of age, gm.	38.9 ± 0.40	51.2 ± 0.17	12.3	0.64
Gain during depletion, gm.	70.7 ± 1.80	54.2 ± 0.57	16.5	2.81
Days to depletion, days	46.3 ± 0.71	36.3 ± 0.15	10.0	1.08
Initial weight of test period, gm.	110.4 ± 1.96	105.9 ± 0.58	4.5	1.94
Gain during test period gm.	51.2 ± 2.45	12.5 ± 1.08	38.7	2.29
Days on experiment, days	52.1 ± 0.77	48.7 ± 0.41	3.4	1.32
Total basal diet during test period, gm.	529 ± 11.8	359 ± 4.7	170	4.94

Section IV

Report of an Attempt to Reduce the Variability of the Weight Gains of Vitamin A Test-Animals by Controlling the Food Intake

In Section I of this thesis it was shown that the amount of basal diet eaten by an animal affected the gain in weight to a much greater extent than any other measured variable and it was suggested that this might be a good point of attack in attempting to produce less variable weight gains among our animals. Using the statistical analysis of our data as a guide, an attempt has been made to devise a method of technic which will control this variable.

Sherman and Burtis (5) have compared the weight gains of small, medium-sized and large test animals when fed the same amounts of vitamin A and conclude the following: "In order to obtain results of the highest accuracy in quantitative determinations of vitamin A by the rat growth method, only such animals as do not differ greatly among themselves in size at the end of the depletion period should be used. For conditions such as those of our laboratory, a minimum limit of 70 to 75 gm. and a maximum limit of 100 gm. at the end of the depletion period would seem to be desirable." The mean weight at the end of the depletion period of all of the vitamin A test rats recorded by Sherman and Burtis is 84 grams. They are recommending then, that animals whose weights at the end of the depletion period range about the mean be used in order to obtain the most accurate results in quantitative tests for vitamin A.

Rather than set arbitrary limits of weight and discard those animals whose weights do not fall within the limits we became interested in the possibility of controlling the weight gains of our animals during the depletion period.

A statistical treatment of the data of the vitamin A depletion period of 123 standard test animals from our Wistar colony was made, and upon the basis of the knowledge thus gained we have attempted to control the weight gains of our animals by controlling the amount of basal diet they consumed. The method we have used was designed to produce animals whose weights at the end of the depletion period approach more closely the mean than the weights of animals fed the basal diet ad libitum.

The means and probable errors for the depletion period of these 123 animals used for vitamin A tests in this laboratory are:

Mean initial weight............	51.9 ± 0.29
Mean gain......................	70.2 ± 0.87
Mean days to depletion.........	35.9 ± 0.33
Mean basal diet eaten daily...	8.66 ± 0.06

The correlations between the variables are:

Initial weight and total gain.................	0.24 ± 0.06
Initial weight and days to depletion...........	0.53 ± 0.04
Initial weight and mean basal diet eaten daily.	0.27 ± 0.06
Total gain and days to depletion..............	0.31 ± 0.05
Total gain and mean basal diet eaten daily.....	0.64 ± 0.04
Days to depletion and mean basal diet eaten daily....................................	0.09 ± 0.06

The largest and most significant of these correlations is that between the total gain and the mean basal diet eaten daily, 0.64 ± 0.04.

A multiple correlation coefficient (R), and a regression equation were calculated according to the method of Wallace and Snedecor (4). The initial weight (A), total gain (B), and days to depletion (C), were used as the independent variables and the mean daily food intake ($\bar{X}$) as the dependent variable. The multiple correlation coefficient (R), is 0.77 ± 0.04. The regression equation is:

$$\bar{X} = 0.07A + 0.05B - 0.09C + 4.56$$

This equation is interpreted to have the following meaning. The mean daily food intake:

(1) Increases 0.07 gms. per unit increase in the initial weight.

(2) Increases 0.05 gms. per unit increase in the total gain.

(3) Increases 0.09 gms. per unit decrease in the days to depletion.

By substituting actual values for A, B, and C in the equation, the mean daily food intake can be calculated. From the standpoint of theory it follows that if the amount of basal diet ingested by the animal is maintained at the estimated mean during the depletion period, and if the variables A and C approximate their means, the gain in weight should also approximate its mean. Thus fewer animals would need to be discarded at the end of the depletion period because they were too large or too small.

Further examination of the data of the 123 records upon which the regression equation was based showed that:

1	rat	of	the	123	was	depleted	between	the	3rd	and	4th	week.
52	rats	"	"	"	were	"	"	"	4th	"	5th	"
52	"	"	"	"	"	"	"	"	5th	"	6th	"
16	"	"	"	"	"	"	"	"	6th	"	7th	"
1	rat	"	"	"	was	"	"	"	7th	"	8th	"
1	"	"	"	"	"	"	"	"	8th	"	9th	"

The mean initial weight of the 52 rats that were depleted some time between the 4th and 5th weeks was 49 grams and the mean gain made by these animals 66 grams. The mean initial weight of the 52 rats that were depleted between the 5th and 6th weeks was 54 grams and their mean gain 71 grams. The mean of the initial weights 49 and 54 is 51 grams. Thus it was decided that an animal weighing less than 51 grams at 28 days of age would be assigned to a depletion period of 4 to 5 weeks and an animal weighing more than 51 grams would be assigned to a depletion period of 5 to 6 weeks. Since the variable C (days to depletion) in the regression equation is expressed in days, 32 days was chosen as the mean depletion period of the one group and 39 days as the mean depletion period of the other.

Thus, if an animal weighed less than 51 grams at 28 days of age, its food intake was estimated by substituting the initial weight for variable A, 66 grams for variable B and 32 days for variable C in the regression equation and solving for $\bar{X}$. $\bar{X}$ is the mean amount of food that should be fed daily in order to produce a gain in weight of 66 grams in 32 days.

Examining the daily food intake records of the original 123 rats it was found that there was a general tendency for the food intake to increase during the first two weeks of the depletion period and then to decrease. In order to take this variation into account averages for each of the two groups, i.e., 4 to 5 weeks depletion and 5 to 6 weeks depletion, were made and food intake curves plotted. These curves are shown in Chart I.

An explanation of the method used can best be explained by a specific example. Suppose that an animal weighed 60 grams at 28 days of age. Since this weight is more than 51 grams the rat would be assigned to the group to be depleted in 39 days and to gain 71 grams. Substituting these values in the regression equation, and solving for $\bar{X}$, the mean daily food intake is found to be 8.8 grams. This quantity of food would then be fed to the animal according to the food intake curve shown in Chart I. A scale of the same dimensions as the abscissa of the curve had previously been made on tracing paper, Chart II. This transparent scale is then placed on top of the curve so that the point 8.8 will coincide with the dotted horizontal line on Chart I which is the mean food intake of the group of 52 rats whose food

Chart I

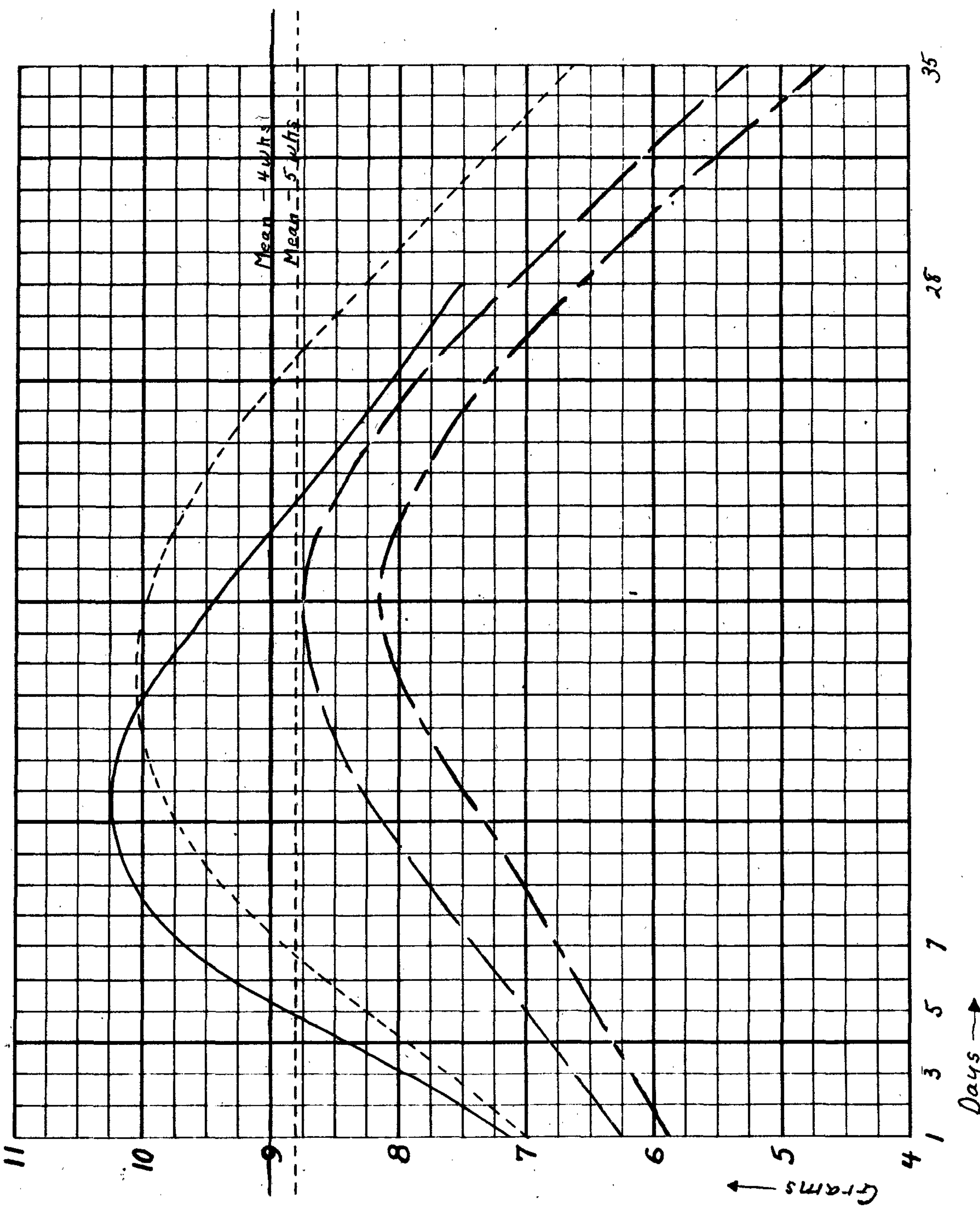
Food intake curves of two groups of 52 rats each during the depletion period of a vitamin A test.

________________ Food intake curve and mean food intake of rats depleted in 4 to 5 weeks.

---------------- Food intake curve and mean food intake of rats depleted in 5 to 6 weeks.

_______ _______ Food intake curve of the 5 weeks depletion group lowered the distance of one standard deviation.

_______ _ _______ Food intake curve of the 5 weeks depletion group lowered the distance of 1.5 standard deviations.


Mean - 4 whs
Mean - 5 whs
11
10
9
8
7
6
5
4
Grams
1
3
5
7
28
35
Days

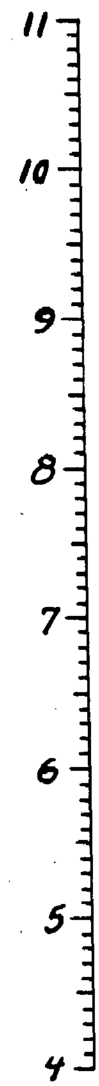


Chart II

Scale used with Chart I in reading the daily allotment of food for each animal.

intake is represented by the curve. Holding the scale at this level and sliding it from left to right the quantity of food to be fed to that particular rat each day may be read at the point where the scale intersects the curve. In this manner the food to be fed to each rat each day can be determined in advance and that quantity weighed out each day and offered to the animal.

The above procedure was followed in all of the succeeding experiments. Five groups of 20 rats each were fed. The first group of 20 animals was fed the basal vitamin A free diet ad libitum to serve as a control group. The second group was comprised of those animals whose initial weights were less than 51 grams. These animals were assigned to a depletion period of 32 days and a gain of 66 grams. The third group was comprised of animals whose initial weights were more than 51 grams and were assigned to a depletion period of 39 days and a gain of 71 grams. For groups two and three the theoretical amount of food was weighed out each day and offered to the animal. If this quantity of food was not eaten in toto it was allowed to remain in the cage. Each week the food left over was removed from the cage, weighed and the weight recorded. In the control group the amount of food eaten daily was determined by subtracting the weight of food left from that offered each day. The performance of these three groups of rats may be read in Tables IX, X, and XI. The theoretical or estimated values are given parallel to the actual values in order to simplify a comparison. In comparing the actual and estimated food intake an allowance of 0.1 gm. to 0.2 gm. must be made for the food spilled.

It will be seen by examining tables X and XI that only eleven rats ate as much as the estimated mean minus 0.6 gm. (P.E. 0.43 gms.; allowance for food spilled 0.2 gms.) According to statistical theory 50 per cent of the animals should have eaten a quantity of food within the range of the estimated mean plus or minus its probable error. Since the animals did not eat as much as this, they were in reality being fed ad libitum and were not different from the control animals.

The above method of controlling the food intake was tested further by another trial. The standard deviations of the weekly food intake records of the original group of 123 rats were calculated. The food intake curve shown in Chart I was lowered the distance of one standard deviation. The fourth group of 20 rats was fed on the basis of this curve. They then received daily a quantity of food equivalent to the original mean minus the standard deviation. The results of this feeding test can be seen in Table XII. In this test the actual food intake approaches more closely the estimated food intake.

A fifth trial was made. This time the food intake curve was dropped one and five-tenths standard deviations so that the animals received the mean food intake minus 1.5σ. The results of this test are shown in Table XIII.

TABLE IX

Weights of Food Intake Together with the Initial Weights, Gains, and Days to Depletion for Each of the 20 Control Rats Fed the Basal Diet Ad Libitum

Animal number	Sex	Initial weight	Gain in weight	Days to depletion	Actual mean daily food intake
		gm.	gm.	days	gm.
1	♀	52	53	42	7.8
2	♂	58	72	41	8.4
3	♀	60	48	35	8.0
4	♀	57	36	32	7.2
5	♂	58	72	34	9.4
6	♀	70	42	35	7.5
7	♂	45	35	30	5.8
8	♀	38	28	31	5.3
9	♀	51	49	37	5.7
10	♂	55	43	32	7.1
11	♂	49	44	28	6.9
12	♂	43	66	36	6.9
13	♀	48	43	36	7.0
14	♀	52	44	36	7.8
15	♂	58	47	35	7.6
16	♀	46	40	35	6.8
17	♂	41	51	32	6.8
18	♀	44	45	32	6.9
19	♀	46	61	35	8.1
20	♀	48	64	42	7.6
Mean and probable error		51.4 ±1.18	49.1 ±1.79	34.8 ±0.55	7.23 ±0.14

TABLE X

Estimated and Actual Weights of Food Intake Together with the Initial Weights, Gains, and Days to Depletion for Each of the 20 Rats Fed the Estimated Mean Food for a 4 Weeks Depletion Period

Animal number	Sex	Initial weight	Gain in weight	Days to depletion	Estimated mean daily food intake	Actual mean daily food intake
		gm.	gm.	days	gm.	gm.
1	♀	55	65	36	9.1	9.0
2	♂	58	62	35	9.3	7.9
3	♀	57	65	37	9.2	8.0
4	♂	70	62	30	10.2	9.0
5	♀	38	33	32	7.8	5.9
6	♀	40	33	32	7.9	6.2
7	♂	44	36	28	8.2	6.8
8	♂	45	37	28	8.3	6.4
9	♂	45	34	28	8.3	6.7
10	♀	42	48	42	8.1	7.5
11	♂	49	49	28	8.6	6.8
12	♀	47	38	35	8.4	6.4
13	♂	50	52	28	8.7	7.5
14	♀	42	50	35	8.1	6.5
15	♂	45	44	35	8.3	7.1
16	♂	47	71	35	8.4	8.3
17	♂	43	55	29	8.1	7.4
18	♀	43	27	35	8.1	6.5
19	♂	43	32	44	8.1	6.1
20	♀	41	41	32	8.0	7.1
Mean and probable errors		47.2 ±1.12	46.7 ±1.94	33.2 ±0.68	8.46 ±0.43	7.15 0.13

TABLE XI

Estimated and Actual Weights of Food Intake Together with the Initial Weights, Gains, and Days to Depletion for Each of the 19 Rats Fed the Estimated Mean Food for the 5 Weeks Depletion Test

Animal number	Sex	Initial weight	Gain in weight	Days to depletion	Estimated mean daily food intake	Actual mean daily food intake
		gm.	gm.	days	gm.	gm.
1	♂	58	48	29	8.9	7.9
2	♀	64	39	30	9.3	7.2
3	♂	63	53	30	9.3	7.9
4	♂	65	77	35	9.4	8.7
5	♀	66	73	42	9.5	9.3
6	♀	70	51	35	9.8	8.0
7	♂	59	49	35	8.9	6.8
8	♀	51	39	37	8.3	6.4
9	♂	55	43	37	8.6	7.5
10	♀	58	71	33	8.9	8.9
11	♀	62	59	33	9.2	8.8
12	♀	58	65	35	8.9	8.6
13	♂	62	62	32	9.2	9.1
14	♂	56	70	33	8.7	8.5
15	♂	61	49	35	9.1	7.6
16	♀	56	68	35	8.7	8.0
17	♀	52	50	38	9.4	7.1
18	♀	54	60	43	8.5	8.2
19	♂	56	73	35	8.7	8.1
Mean and probable error		59.2 ±0.76	57.8 ±1.82	34.8 ±0.55	8.96 ±0.43	8.02 0.12

TABLE XII

Estimated and Actual Values of Food Intake Together with the Initial Weights, Gains, and Days to Depletion for Each of the 20 Rats Fed the Estimated Mean Food Minus the Standard Deviation for a 5 Weeks Depletion Test

Animal number	Sex	Initial weight	Gain in weight	Days to depletion	Estimated mean daily food intake	Actual mean daily food intake
		gm.	gm.	days	gm.	gm.
1	♂	58	42	35	7.7	6.7
2	♀	53	39	38	7.4	6.7
3	♂	62	44	35	7.9	6.9
4	♀	54	28	33	7.4	5.9
5	♀	54	23	35	7.4	6.1
6	♀	55	22	43	7.5	6.3
7	♂	66	54	30	8.3	8.0
8	♂	56	49	31	7.5	7.1
9	♀	66	53	35	8.3	7.9
10	♂	55	43	33	7.5	6.8
11	♀	68	44	35	8.5	7.8
12	♂	52	39	35	7.2	5.9
13	♂	51	51	32	7.1	6.9
14	♂	52	35	31	7.2	6.5
15	♂	58	56	35	7.7	7.4
16	♂	59	52	35	7.8	7.5
17	♀	61	54	39	7.9	7.8
18	♀	64	54	39	8.2	8.0
19	♀	56	40	42	7.5	7.3
20	♀	58	47	38	7.7	7.5
Mean and probable error		57.9 ±0.75	43.4 ±1.51	35.4 ±0.51	7.68 ±0.43	7.05 ±0.08

TABLE XIII

Estimated and Actual Values of Food Intake Together with the Initial Weights, Gains, and Days to Depletion for Each of the 20 Rats Fed the Estimated Mean Food Minus 1.5 Standard Deviations for a 5 Weeks Depletion Test

Animal number	Sex	Initial weight	Gain in weight	Days to depletion	Estimated mean daily food intake	Actual mean daily food intake
		gm.	gm.	days	gm.	gm.
1	♀	51	16	35	6.5	6.0
2	♂	55	44	35	6.8	6.2
3	♂	59	45	35	7.2	6.5
4	♂	58	48	35	7.1	6.9
5	♂	52	49	35	6.6	6.4
6	♂	52	49	35	6.6	6.4
7	♀	53	46	35	6.7	6.5
8	♂	53	47	35	6.7	6.3
9	♀	52	44	29	6.6	6.8
10	♀	56	42	33	6.9	6.9
11	♂	60	54	35	7.3	7.1
12	♂	54	50	32	6.8	6.7
13	♂	56	40	35	6.9	6.7
14	♀	51	58	40	6.5	6.4
15	♂	55	69	39	6.9	6.8
16	♀	54	58	40	6.8	6.6
17	♀	56	40	37	6.9	6.8
18	♀	53	63	38	6.7	6.6
19	♀	51	51	40	6.5	6.4
20	♂	54	71	39	6.8	6.7
Mean and probable error		54.2 ±0.39	49.2 ±1.74	35.8 ±0.42	6.79 ± 0.43	6.58 ±0.04

It was hoped that the limitation of the quantity of food by this method of feeding would lower the variability in the weight gains made during the depletion period. The standard deviations of the weight gains of the five groups of 20 rats each are as follows: Control group 11.86, four weeks depletion 12.86, five weeks depletion 11.79, five weeks depletion fed M - σ 10.00, and five weeks depletion fed M - 1.5σ, 11.51. The differences in the variance of the groups is quite insignificant. The method of testing this difference was that recommended by Fisher (11).

Other methods of controlling the food intake of experimental animals have been reported in the literature. Steenbock (12) has tried limiting the food intake of the entire group of rats to that quantity eaten by the animal eating the least amount. This method has its disadvantages as the entire group may be starved unduly because one rat suffers an inappetence which may or may not be due to the dietary treatment under investigation. Mitchell (13) recommends the paired feeding method and reports considerable success in its use. Morris and Palmer (14), however, report an individual variation in their animals large enough to render the paired feeding method inadequate in attempting to evaluate small dietary differences.

If we compare the mean initial weight, mean gain, mean days to depletion and mean daily food intake of the 59 test animals reported in Tables IX, X, and XI with the means of the original

123 animals we see that the differences in mean initial weights and days to depletion are not significant, but the differences in mean gain and mean daily food intake are significant. Evidently the 59 test animals and the original 123 animals do not belong to the same population as far as these two factors are concerned.

A study of the seasonal variation between the two groups and a study of the litter size was made in an attempt to explain the change in population. Five of the original 123 rats came from litters of nine rats, 85 from litters of eight, seven from litters of seven, 24 from litters of six, and two from a litter of five. Of the 59 test rats, nine came from litters of nine, 27 from litters of eight, eight from litters of seven, ten from litters of five, two from litters of four, and three from litters of three. Since the distribution of the animals in the different sized litters is approximately the same the litter size is probably not a factor. The 59 test rats were either sons and daughters or grandsons and granddaughters of the same stock colony as the original 123 animals. The colony has been inbred for fifty generations by strictly brother and sister matings. There has been no variation in the diet of either the stock colony or of the experimental animals.

Ten of the 123 rats upon which the regression equation was based were started on experiment in January 1929, 17 rats were started on experiment in February, 42 in March, 12 in April, 11 in May and 31 in June of the same year. Of the 59 test rats, 11 were started on experiment the last of December 1929, 38 were started in January 1930 and ten in February 1930.

The original 123 animals were divided into two groups, the animals started on experiment in January, February and March of 1929 making one group, and those started in May and June of 1929 a second group. The 59 test animals were started during the succeeding winter and form a third group. The means of the measured variables together with the mean differences and their standard deviations may be read in Table XIV. The differences in the mean initial weights mean days to depletion are not significant. The differences in the mean gains are all significant. Two of the differences in food consumption are significant. The third difference, i.e., that between the rats fed during the summer 1929 and winter 1930, approaches significance, the mean difference being 2.1 times its probable error. These differences lead one to conclude that the seasonal variation is not the factor responsible for the changes in the population. Rather there seems to be a progressive change within the colony itself.

This change in the population of our colony is exceedingly interesting and important. It means that comparative vitamin tests must be run parallel to each other in order to eliminate in so far as possible variations due to a changing population of animals. It also means that we are in the habit of considering the so-called standard test animal more homologous than it really is.

TABLE XIV

Means of the Measured Variables, Mean Differences and Their Standard Deviations for Rats Fed at Different Seasons of the Year

	Mean Initial weight	Mean Gain	Mean Days to depletion	Mean Daily food intake
65 rats fed during the winter 1929	51.2 ± 0.51	73.6 ± 1.28	33.9 ± 0.61	9.10 ± 0.12
42 rats fed during the summer 1929	53.6 ± 0.88	64.9 ± 0.73	37.0 ± 1.29	7.85 ± 0.09
59 rats fed during the winter 1930	52.5 ± 0.73	51.1 ± 1.15	34.2 ± 0.35	7.46 ± 0.08

Mean Differences and Their Standard Deviations

	Initial Wt.		Gain		Days to Depletion		Food Intake	
	Difference	σ	Difference	σ	Difference	σ	Difference	σ
Winter 1929 vs summer 1929	2.4	1.6	8.7	2.2	3.1	2.1	1.25	0.22
Summer 1929 vs winter 1930	1.1	1.7	13.8	2.0	2.8	1.9	.39	0.18
Winter 1929 vs winter 1930	1.3	1.3	22.5	2.5	0.33	1.06	1.6	0.22

Undoubtedly the quantity as well as the quality of the food ingested is an important factor in any study based upon the growth response of an animal. The basal metabolic rate may be an important factor also. Likewise the kind of growth made is a factor, whether it is a retention of water in the tissues, a deposition of fat, or a growth of the bones. The effect of activity upon the efficiency of food utilization is still another unsolved question. Morris (15) has been studying the inheritance of the efficiency quotient of the rat, the efficiency quotient being expressed

$$\frac{\text{Digestible dry matter consumed}}{\dfrac{\text{Gain in Weight}}{\text{Mean weight during experiment}} \times 100}$$

as, (16)

He has developed two strains of rats, one with a high and one with a low efficiency quotient. Perhaps by selective breeding, strains of rats can be developed in which the physiological functions mentioned above will be much more uniform than they are at present. We may then expect more consistent reactions in biological tests and will have an animal which can more properly be termed "standard".

SUMMARY

An application of statistical method to the data of 469 vitamin A feeding tests was made with a view to determining the factors influencing the weight gains of the test animals. The data of this study indicated a possible difference in the reactions of rats fed vitamin A free diets containing different amounts of fat. The data showed also that the quantity of basal diet ingested was the measured variable having the greatest per cent effect upon the weight gains of the animals.

An experiment was conducted to test the difference in the reactions during the depletion period of vitamin A test-animals fed diets containing and not containing fat. One hundred and twenty-three animals were fed the basal diet containing fat and 60 the fat-free basal diet. The difference in the mean gains in weight was not found to be significant but that between the number of days to depletion was significant. From these data one may conclude that a vitamin A free diet containing fat is preferable to a fat free basal diet as it shortens the time required to deplete the body store of vitamin A.

An analysis of the reactions of vitamin A test animals from three different stock colonies revealed significant differences showing that the results of this or any other study could not be applied directly to the animals of any other colony without first testing the homogeneity of the two colonies.

Since the food intake was shown to be the factor having the greatest per cent effect upon the gains in weight of the

test animals, an attempt was made to regulate their gains by controlling this factor. An estimate of the food intake was made by means of a regression equation in which the mean initial weight, mean gain, and mean days to depletion were used as the independent variables and mean daily food intake as the dependent variable. Five groups of 20 rats each were fed a quantity of the basal diet estimated by the regression equation. This method of feeding the animals did not result in less variable weight-gains. In analysing the data to discern reasons for the failure of these 100 animals to react positively to the test it was discovered that the test animals and the 123 animals upon which the regression equation was based did not belong to the same population even though both groups were offspring of a highly inbred colony of rats.

The reactions of animals fed during three consecutive seasons, winter 1929, summer 1929 and winter 1930 indicated that there was a progressive change in the amounts of the basal diet eaten and in the weight gains made. This argues for the necessity of standardizing the animals in regard to these two factors and shows that until more uniform reactions can be ascertained comparative vitamin tests must be run simultaneously in order to prevent such changes in the population from vitiating the results of the experiment.

CONCLUSIONS

(1) The quantity of basal diet ingested by vitamin A test-animals is the measured variable having the greatest per cent effect upon the gains in weight.

(2) The presence of fat in a vitamin A free diet is desirable as it shortens the number of days required to exhaust the vitamin A stored in the body of the rat.

(3) The results of a vitamin study made upon one stock colony can not be applied directly to the animals of another colony without first testing the homogeneity of the two colonies.

(4) There seems to be a progressive change in the reactions of the vitamin A test animals of this colony. This means that comparative vitamin tests must be run simultaneously in order to prevent such changes from vitiating the results.

(5) The progressive change in the population of the vitamin A test animals observed suggests that their reactions might be standardized by selective breeding. This appears to be the most promising field for further study.

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