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THE EFFECT OF THE BACTERIAL
FLORA ON THE BIOLOGICAL
TEST FOR VITAMINE B

THESIS

Submitted to the Graduate
Committee in candidacy for
Degree of

MASTER OF SCIENCE.

by

Bertha Garlock

1923

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A thesis submitted to the Graduate
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Major subject Biological Chemistry

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The Effect of the Bacterial Flora on
the Biological Test for Vitamine B

Despite the fact that the existence of vitamins has been recognized for a considerable period of time and that an enormous amount of experimental work has been reported in practically every scientific journal, there has not been found, to date, a single recognized qualitative or quantitative test for their presence. The biological test remains today, as it was in the beginning, the only method of testing for these substances. Until the chemist is successful in his endeavors to isolate and analyze the vitamins, we must continue the use of this method, slow and unsatisfactory as it is.

Through the findings of Steenbock, Sell and Nelson (1) considerable discredit has accrued even to the biological tests. While studying the vitamine B level of rations, these investigators found, 1923, that rats reared in the usual laboratory manner grew and developed normally when fed on a certain standard synthetic ration,

but when placed upon screens so they did not have access to their feces, and when given the same ration, they failed to grow. This was shown to be due to a deficiency in vitamine B, for when this substance was added to their ration, they grew and developed normally. This same phenomenon had been observed and reported by Mendell and Osborne (2) in 1911, but they made no attempt to explain it.

Some investigators seem to question the importance of this discovery, but there is no doubt that the growth curves in the early stages of feeding are somewhat influenced when the animals are allowed access to their feces. The questions then suggest themselves: First, is the better growth of the rats that have access to their feces produced by the vitamine which has passed through the bodies of the animals only partially utilized? Second, is the vitamine a catalytic substance that is not destroyed by the process of digestion and is it therefore capable of producing its beneficial effects time after time? Third, may it be that the

bacteria which grow in the intestinal tract of the animals synthesize vitamine B and store it within their own bodies? The latter theory is worthy of consideration, as it has been demonstrated by numerous investigators that certain microorganisms have the ability to synthesize vitamine B when grown upon media known to be free from this constituent. Damon (3), in his work with the acid-fast bacteria, has demonstrated that three members of this family were capable of synthesizing vitamine B: namely, *B. timothy*, *B. senegalis*, and *B. Moelleri*. The same investigator (4) proved also that a mucoid organism, Pfeiffer's bacillus, could synthesize and store vitamine B. Portier (5) asserts that mitochondria, which he also calls symbiotes, are merely bacteria adapted to the cell contents and are capable of synthesizing vitamine B. Nelson, Fulmer, and Cessina (6) have demonstrated the same for yeast. On the other hand, Damon (7) demonstrated also that *B. Coli*, *B. paratyphosus*, and *B. subtilis* did not produce vitamine B. He also proved the same for the spore-bearing aerobic

organism, *B. adnaerens*, and the mucoid organism, Friedlander's bacillus.

In an attempt to determine the facts connected with the third theory mentioned above and to study the change in the bacterial flora of the intestinal tract, the following work was undertaken.

EXPERIMENTAL

Animals weighing approximately fifty grams were selected from carefully reared stock for these experiments. In an effort to offset any litter individualities the rats were divided so that each cage used for comparative purposes contained one animal from the same litter. Six groups were used in the initial experiment. Cages numbered One, Two, and Three were round wire cages, fourteen inches in diameter, set over tin bottoms of the same size; cages numbered Four, Five and Six were the same type but were fitted with bottoms of hardware cloth of one-fourth inch mesh. This false bottom was suspended about two inches above the tin bottom thus permitting the feces to fall through into the tin pan below, so the animals had

no access to them.

The basic vitamine-free ration used for all of these determinations was composed of the following:

Dextrin	76.3%
Casein	15.0%
Butter fat	5.0%
Salt mixture	3.0%

The dextrin used in the preparation of these rations was prepared by hydrolyzing starch with one percent of citric acid for three hours under thirty pounds pressure. The casein used was prepared in the laboratory by a modification of the Van Slyke method (8): pure, sweet, fat-free milk was placed in a container fitted with a mechanical stirring device. A mixture composed of one part hydrochloric acid to two parts acetic acid was used as a precipitating agent. The acid was introduced into the milk below the surface, the tip of the tube carrying the acid being so arranged that it was close to the mechanical stirrer and near the bottom of the vessel containing the milk. Introduced in this way, the acid does not produce a curd where it first comes in

contact with the milk. The acid was run in slowly until 60 cc per liter had been introduced. The mixture was then allowed to stand three hours with gentle stirring, when about 30 cc more of the acid were added while the mixture was being stirred rapidly. A gentle heat was then applied to make the curd more firm and thus to facilitate the separation of the curd from the whey. The warmed mass was then poured out on a cheesecloth stretched over a screen, and allowed to drain over night. The following morning the casein, now practically free from any excess milk, was washed with distilled water, placed in a jar, and covered with water containing a small amount of acetic acid; this water was changed each day for a period of six weeks in order to remove the salts and vitamine B. The casein was then washed with distilled water, dried, and ground ready for use. The butter was filtered in the usual manner through a hot funnel to remove the water and salts present. The salt mixture was McCollum's number 185 (9).

Two animals, a male and a female, were placed on shavings in cage No. 1. They were fed the basic ration which was supplemented by five percent of yeast in place of an equal amount of dextrin. The other cages contained two males and two females each. The animals in cage No. 2 were supplied with shavings for bedding. In cage No. 3 they were given no shavings but were placed on pans so they had access to their feces. The animals in cages No. 4, No. 5, and No. 6 were placed on wire screens so they had no access to their feces. All were then fed the basic vitamin-free ration, those animals in cage No. 5 having access to a cup of shavings placed in their cage as a source of roughage, while those in cage No. 6 had the ration supplemented by five percent of agar-agar, to take the place of the five percent of dextrin which had been removed. All the animals had an abundant supply of distilled water.

The animals were weighed weekly and the growth curves plotted. Typical curves are shown by the accompanying charts.

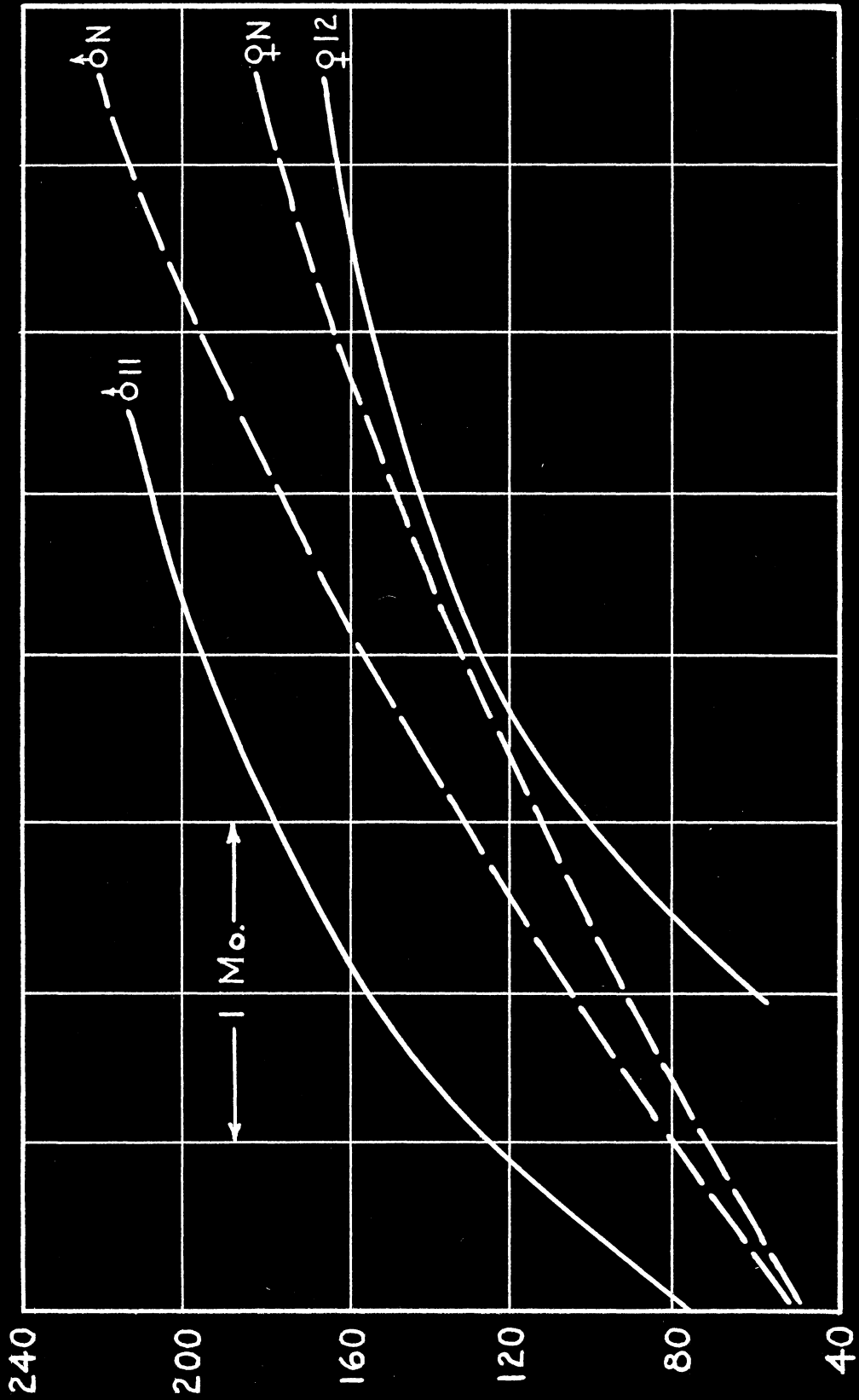


CHART I

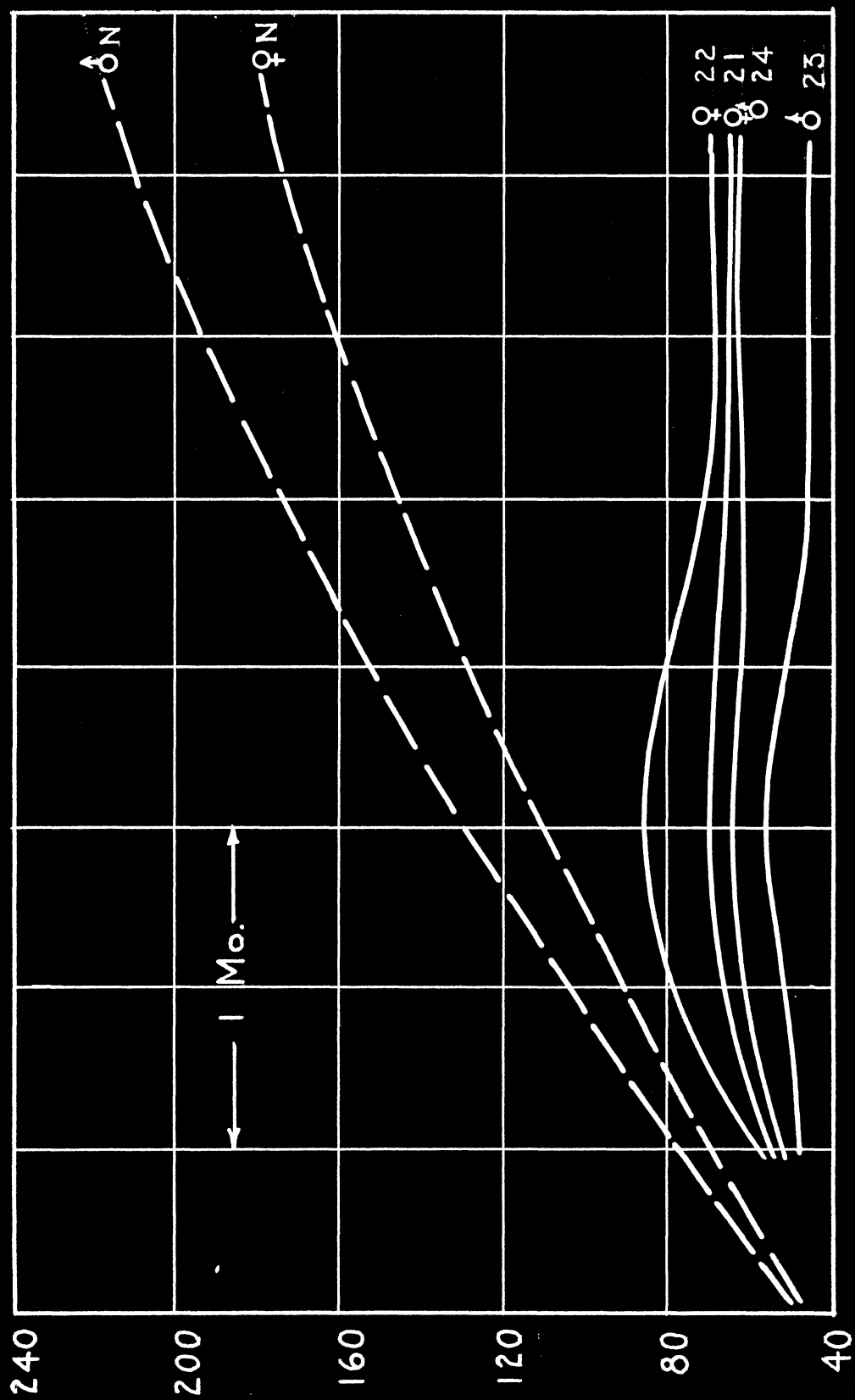


CHART II

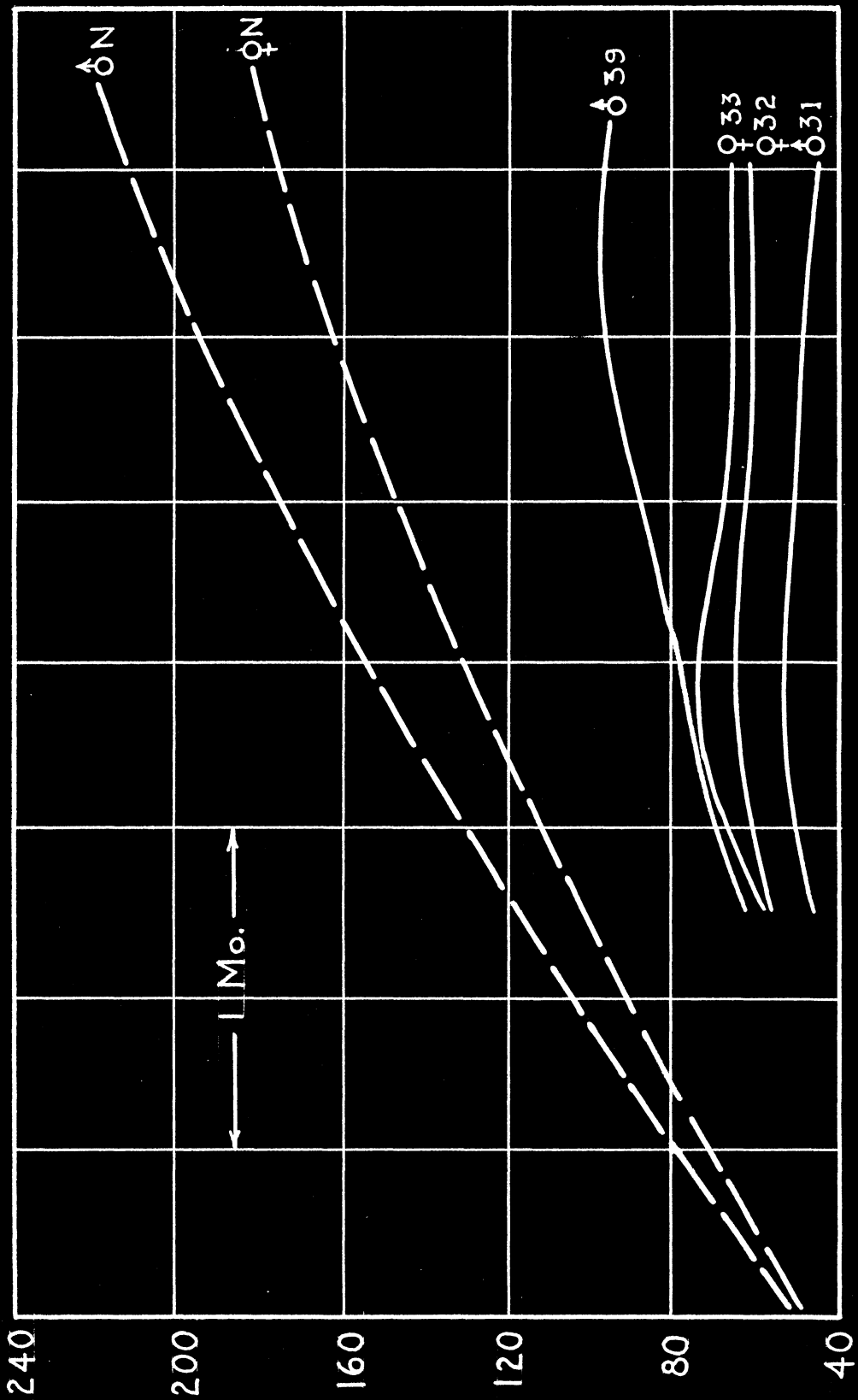


CHART III

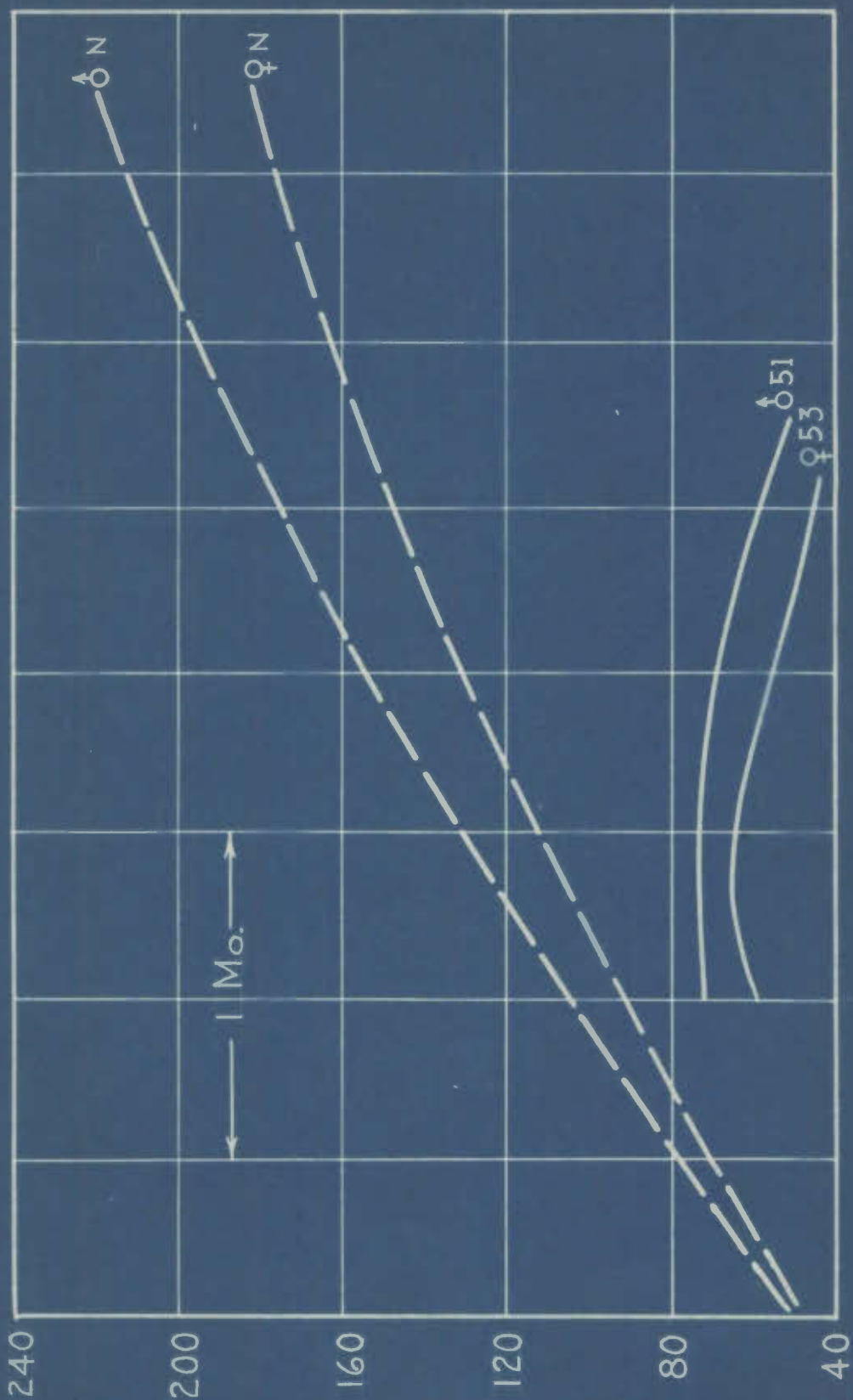


CHART V

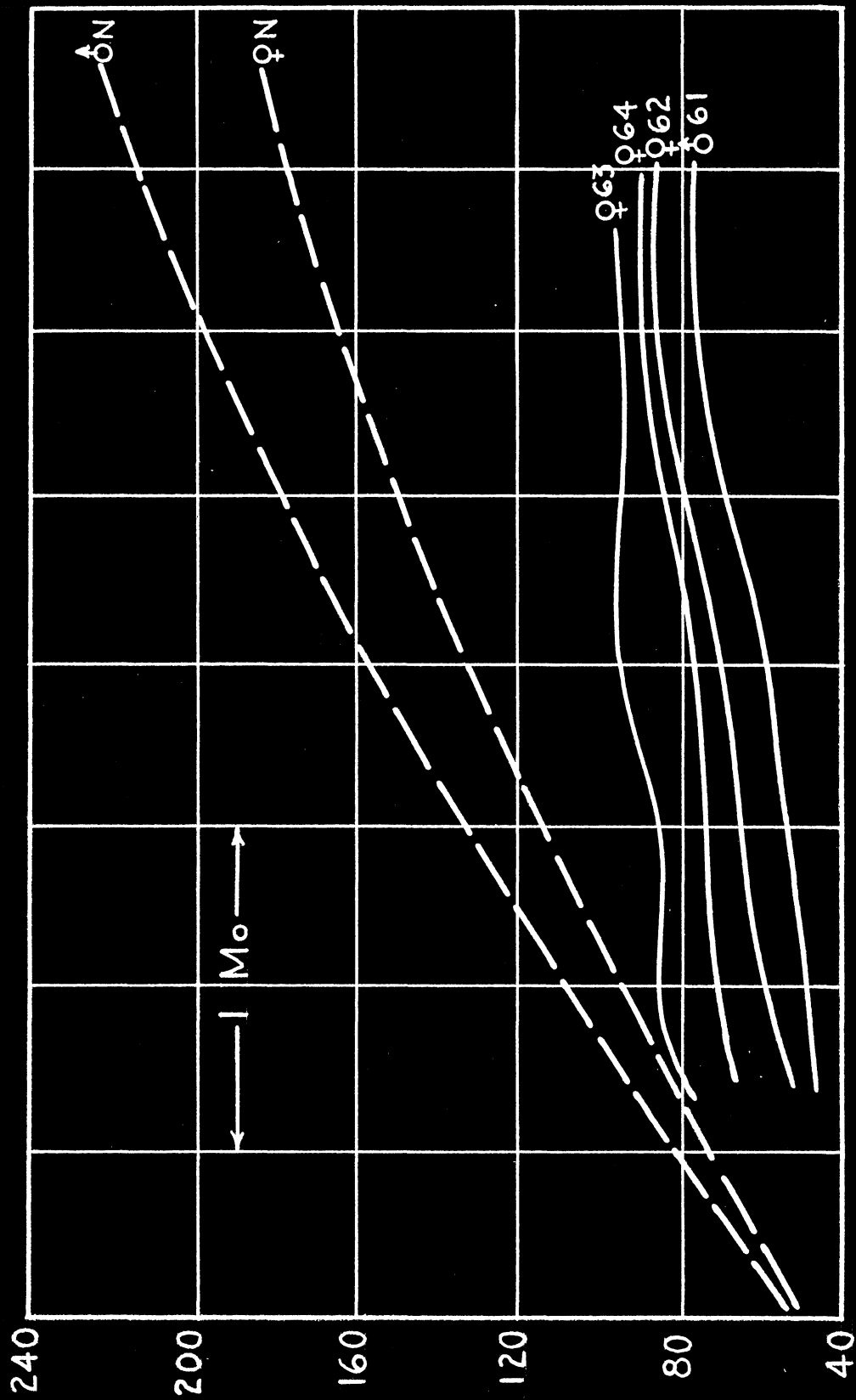


CHART VI

CHART VII

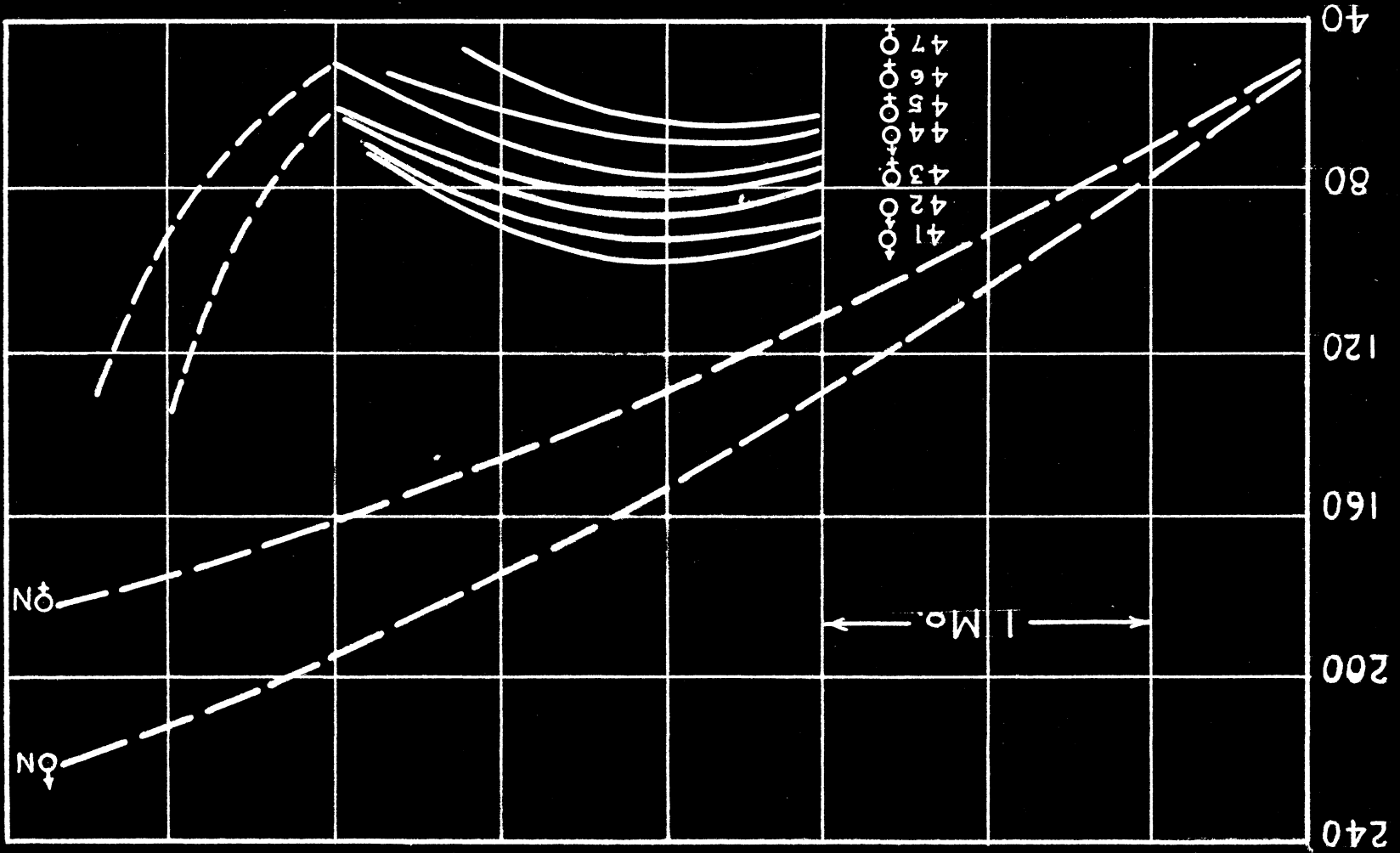


Chart I shows the curves of animals 11 and 12, from cage No. 1, those having the basic ration supplemented by five percent of yeast. These curves are normal in every way, and show that outside the vitamine content, this ration is entirely adequate for growth. Chart II represents the animals from cage No. 2, which were fed the basic ration and bedded with shavings. Chart III shows the growth of the animals in cage No. 3, which were placed on pans without shavings. Chart VII shows the curves of the animals in cage No. 4, which were placed on screens and so had no access to their feces. Charts V and VI respectively, show the curves of the animals placed on screens with access to a limited supply of shavings, and of those having five percent of agar-agar added to their diet in place of a similar amount of dextrin. An inspection of these curves show that growth ceases and death overtakes the animals much sooner when they do not have access to their feces. This is shown in comparing the curves of animals 31, 32, 33, and 34 in chart III with the

solid curves of animals 41, 42, 43, 44, 45, 46, and 47 in chart VII. The animals shown on chart III that had access to their feces lived a much longer period. They grew for practically the same period of time, but their decline was more gradual. When they reached a point a little above their original weight, they seemed to reach a level at which they were able to maintain their weight practically constant for several weeks, showing that in some way they must have been gaining a limited supply of the necessary vitamins during a portion of the time. After the animals in cage No. 4 died, four more animals were placed in the cage and fed the same ration under the same conditions, with the result that two of them died during the seventh week, at which time the feces from this cage which had been collected and dried to prevent bacterial action, were ground and added to the ration. It was found that when this material made about twenty percent of their ration, the two remaining animals grew and developed rapidly as shown in the dotted extensions to the

curves of animals 44 and 45, chart VII. It is noticeable then that several factors must be concerned in these curves, as the animals in cage No. 5 that had even shavings as a source of roughage, fared better than those without, while the addition of five percent of agar-agar produced a curve somewhat different from those for the rats in any of the other cages. They not only made a better growth but also failed to decline as rapidly as those that did not have the agar-agar. This shows one of two things: either that other factors are involved besides the benefit from eating the feces, or that the agar-agar contains a trace of vitamine B. Large amounts of the agar-agar were extracted with boiling alcohol and the alcoholic extract added to the ration. No beneficial results were obtained, thus indicating that the increased bulk in the intestinal tract was beneficial to the animal.

In an effort to determine the effect of the ration on the bacterial flora of the intestinal tract of the rat and to find, if possible, whether or not any organisms were present which might act

as a synthesizer of vitamin B, the following procedure was carried out: at the end of the first week of the experiment feces from the animals in each cage were collected directly from the animal, under sterile conditions. This material was weighed and transferred to sterile water blanks, thoroughly macerated, and dilution of 1-100, 1-1000, and 1-10000 were made. Plates were poured using one cc and 0.1 cc of each dilution. These were incubated for forty-eight hours. Some of the original dilution from each sample was placed in the fermentation tubes containing lactose broth to determine directly if any members of the intestinal group were present. The formation of gas in each case indicated the presence of this group. After incubating forty-eight hours, the colonies on the plates were studied and counted, the comparative numbers of the various types of organisms per gram of feces being thus determined. The colonies were then transferred to agar slopes and allowed to incubate forty-eight hours. At the end of this

period the cultures were mounted to determine their morphology. Gram's stain, hanging drop, and sugar media were used as an aid in identification. Each week the samples were collected and studied as described above. The comparative numbers of the organisms in thousands per gram of the sample are shown on the following chart:

The figures given are the average taken from several repetitions of the experiment. Figures for cages No. 1 and No. 5 are not shown, as they had no bearing on the findings. No figures for cage No. 4 are available for the seventh week, as either the animals had died before that time or the rations had been changed.

An attempt has been made to establish a relation between the growth curves of the various lots and the prevalence of the bacteria. Undoubtedly there are several factors involved. Both cages No. 2 and No. 3 had access to their feces, but it has been found that those having access to the shavings as well, do not consume as many of their feces as do those without the shavings. The roughage here seems to be somewhat beneficial, as

Week	Cage No. 2	Cage No. 3	Cage No. 4	Cage No. 6	
First	Spore-bearers	77	1,100	15	19
	Non-spore-bearers	479	2,535	627	767
Third	Spore-bearers	-	-	-	-
	Non-spore-bearers	333	6,871	490	456
Fifth	Spore-bearers	-	-	-	-
	Non-spore-bearers	600	10,500	1,575	638
Seventh	Spore-bearers	-	-	-	-
	Non-spore-bearers	800	22,000	-	1,360

Chart showing thousands of organisms per gram of feces.

it was also in the case of those fed on screens with access to shavings. In both cages No. 2 and No. 3 the number of spore-bearing organisms was far in excess of the number found in the cages where the animals were kept on screens. In the same manner the growth curves did show the drop as found in the case of the animals kept on screens. The reason for this is thought to be that the spore-bearing organisms are synthesizers of vitamin B. The prolongation of the growth curve was proportional to the number of the spore-bearing organisms. It is also probable that the effect of the roughage in cage was a factor in the growth curves. The animals in cage No. 4 that had no access to the feces showed the fewer spore-bearing organisms per gram of feces than those under other conditions. This would indicate that access to the feces tended to increase the number of spore-bearing organisms in the feces. It is also noteworthy that the number of spore-bearing organisms dropped very rapidly, all having disappeared by the close of the third

week. This may be explained through the development of autolytic products. The non-spore-bearing organisms, as in the case of the spore-bearers, were found in greater numbers in cage No. 3 where the animals had access to their feces; and in much smaller numbers in cage No. 4 where the animals were on screens. The organisms in all cases, except in cage No. 3, decreased in numbers during the third week. In cage No. 3 they had increased greatly. This increase was noticed in all the cages after the fifth week, and was probably due to the greater concentration of the feces, which was caused by the loss of appetite on the part of the animals on the vitamine-free ration.

From a survey of the literature previously mentioned it was found that the *B. coli* group does not synthesize vitamine B, so it was deemed advisable to study the spore-bearing organisms. An attempt was made to grow these organisms on vitamine-free media, to secure a quantity sufficient to add to the vitamine-free ration and thus deter-

mine whether or not they synthesize and store vitamin B. Pure cultures of these organisms were obtained by direct transfers. These organisms were inoculated into cultural media composed of the following:

Beef extract	0.3 gr.
Difco peptone	0.5 "
NaCl	0.1 "
Glycerine	4.5 "
Distilled water	100.0 cc.

Damon(3) in his work with the acid-fast bacteria demonstrated this media to be free from vitamin B. After being allowed to incubate for three days, the growth was filtered, washed, and dried. Ten percent of this material was added to the standard vitamin-free ration and fed to the animals which had ceased to grow on the basic ration. The resulting growth was studied, and curves plotted on Chart VIII.

From a study of this chart it will be seen that when the organisms were added to the ration, they produced an increased growth, shown by the dotted extensions to the growth curves of animals

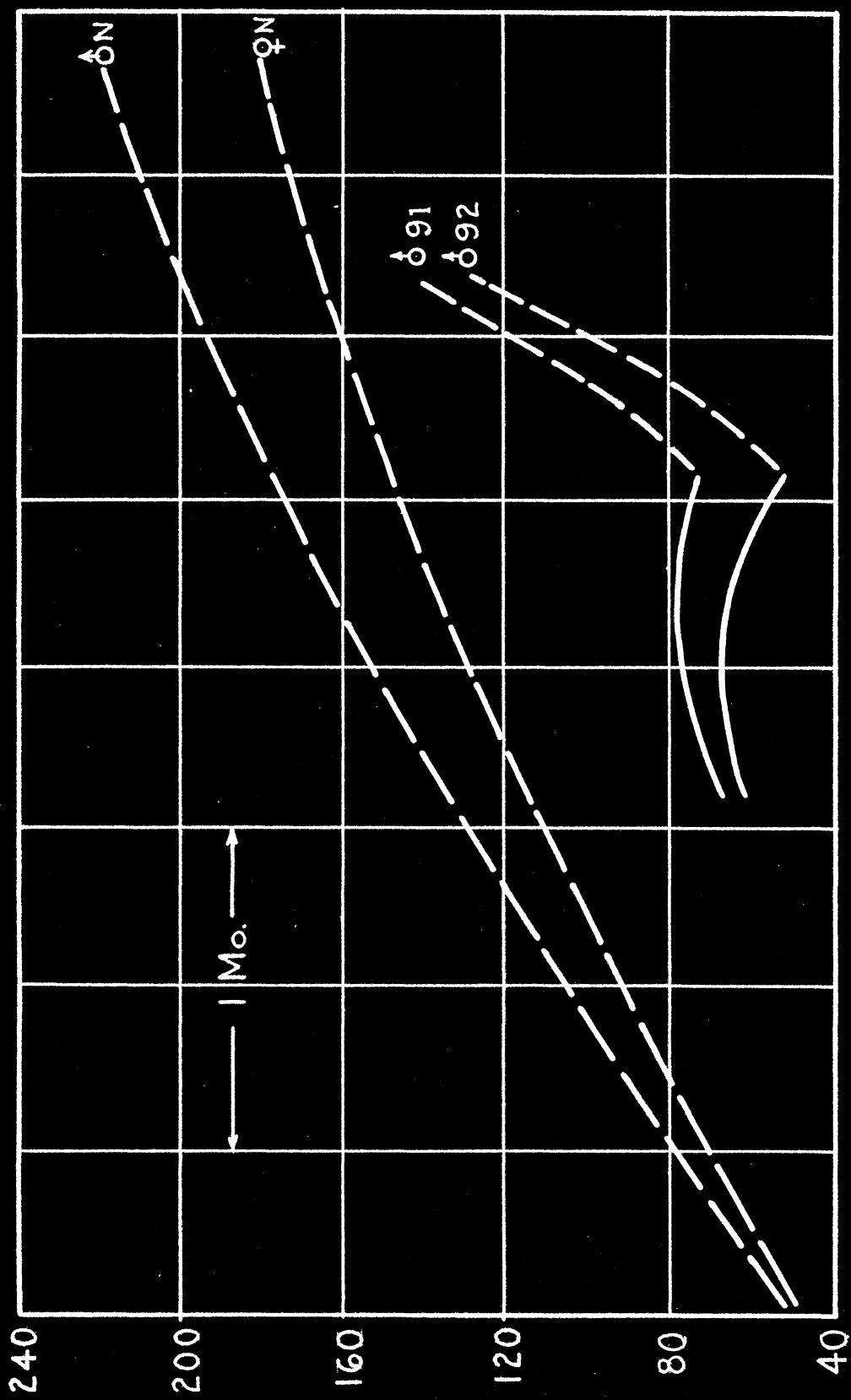


CHART VIII

91 and 92. This indicates that vitamin B is synthesized by the spore-bearing organisms that occurred in the intestinal tract of the rat during the early part of the experiment.

These experiments have been repeated until there seems to be little doubt that there is a correlation between the synthesis of vitamin B by the spore-producing organisms and the extended growth period of the animals. From a close examination of rats fed many types of rations, both on screens and on shavings, it seems that the final results of the biological test are not different under the two conditions. However, the access to shavings and feces retards the final results by several weeks and it would be undesirable to make comparative data between animals fed under the two conditions.

SUMMARY

(1) Experimental animals kept under the usual laboratory conditions do not respond as

quickly to vitanine-free rations as do those given the same rations and placed upon screens.

(2) Feces fed to animals that have ceased to grow on the experimental ration will produce an accelerated growth.

(3) The spore-bearing organisms present in the intestinal tract during the early part of the experiment synthesize and store vitanine B.

(4) Animals kept on pans so they have access to their feces have greater numbers of these spore-bearing organisms during the early part of the experiment which condition is due to the re-ingestion of these organisms. The continued growth of these animals is undoubtedly due to the additional vitanine B which has been synthesized and stored by these spore-bearing organisms.

(5) Animals given roughage in some form make a better growth than those without this material. This is especially true when agar-agar is incorporated in the ration.

(6) Extracts of the various forms of roughage used indicate that the substances contain no vitamins, but produce a better physical condition in the animals.

ACKNOWLEDGEMENT

The writer desires to express thanks to V.G. Heller for assistance in suggesting the problem, and for outlining and supervising the work; to C.H. McElroy of the Bacteriology Department for kindly supervising the bacteriological portion of the work; to O.M. Smith of the Chemistry Department and C.H. McElroy of the Bacteriology Department for supplying the materials for carrying on the experimental work; and Grace Clause for valuable assistance in the routine bacteriological work.

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