THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Examination

This is to certify that we the undersigned, as a committee of the Graduate School, have given Anne Gertrude Benton final oral examination for the degree of Master of Arts . We recommend that the degree of Master of Arts be conferred upon the candidate.

Minneapolis, Minnesota

June 1 1918

W. P. Kirson
Chairman

E. J. Bell.

A. H. Sanford

H. Gortner

C. J. V. Pettibone

THE UNIVERSITY OF MINNESOTA

GRADUATE SCHOOL

Report

of

Committee on Thesis

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by Anne Gertrude Benton for the degree of Master of Arts. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts.

L. P. Larson
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Committee on Thesis

The undersigned, acting as a Committee of the Graduate School, have read the accompanying thesis submitted by Anne G. Benton for the degree of Master of Arts. They approve it as a thesis meeting the requirements of the Graduate School of the University of Minnesota, and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Arts.

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Chairman

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E. C. Rosenow

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THE PROTEOLYTIC ACTIVITY OF VARIOUS PATHOGENS

A Thesis submitted to the

Faculty of the Graduate School of the

University of Minnesota

by

Anne G. Benton

In partial fulfilment of the requirements

for the degree of

Master of Arts

June

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The importance of various protein-decomposition products resulting from bacterial activity was early recognized, both from the point of view of test-tube differentiation of organisms and from that of the possible toxicity of such substances when formed in the body, or ingested. The great mass of investigations of ptomaines and other poisons in food has indirectly contributed much to our understanding of the protein metabolism of microörganisms. At first such studies had to do merely with the identification of the end products of the action of mixed cultures on putrescible materials and were not necessarily controlled as to the exact chemical constituents of the media or the strains of bacteria employed. These researches throw light on the possible or probable decomposition of food-stuffs in the intestine, where several varieties of bacteria are always present, and also show that the formation of certain substances such as ammonia, hydrogen sulphide, skatol, and so forth is characteristic of putrefaction irrespective of the precise nature of the protein or the particular varieties of organisms present. Abderhalden and his associates (1), Ackermann (2), and Neuberg (3) used mixed cultures obtained from putrefying pancreas on known nitrogenous substances in order to observe the end products of decomposition. Berthelot and Bertrand (4, 5, 6) inoculated mixed cul-

1. Abderhalden, Fromme & Hirsch. Zeitschr. f. Physiol. Chem. Vol. 85, p. 131
   Abderhalden & Andor Fodor. Zeitschr.f.Physiol.Chem. Vol.85,p.131, 112.
2. Ackerman. Zeitschr.f.Physiol.Chem.Vol.56,p.87;Vol.65,p.504.
3. Neuberg. Biochem.Zeitschr. Vol.18,p.424,431,435;Vol.37,p.490.
4. Berthelot. C. r. Acad. de Sciences. Vol.153, p.306.
5. Berthelot & Bertrand. C.r.Acad.de Sciences, Vol.156,p.1027; Vol.154,p.1643; Vol.154, p. 1826.
6. Berthelot & Bertrand. C.r.Soc.Biol. Vol. 71, p. 232.

tures of fecal bacteria on inorganic media enriched with a single amino acid, in the hope of isolating strains fermenting by preference the acid in question.

Productive of much more knowledge of actual bacterial metabolism was the long list of researches using pure cultures on media of known composition, making tests to determine the nature of the compounds formed. The indol test is the best known and most useful result of the investigations carried out upon peptones or other tryptophane-containing media. Herter & Broeck (7) using a 1% preparation of casein with B. proteus demonstrated the presence of primary amines, hydrogen sulphide, fatty acids, aromatic oxyacids, indol, and indol acetic acid. Nawiasky (8) compared the products used up by B. proteus on a medium rich in peptone with those assimilated by Vibrio Finkler, B. fecalis alkaligenes, and B. mesentericus, and states that B. proteus attacks albuminoses much more actively than do the others, but that its action on peptones and creatin was less marked. Rettger (9) planted B. putrificus Bienstock, B. edematis maligni, B. anthracis symptomatici, B. coli, and B. lactis aerogenes on his special egg-meat medium and tested for indol, skatol, phenol, aromatic oxyacids, skatol-carbonic acid, tyrosin, leucin, albumoses, peptones, tryptophane, hydrogen sulphide, and mercaptan, his conclusion being that only the obligate anaerobes cause true putrefaction. Later with Newell (10) he tried twenty-six strains of B. proteus on egg-meat medium, blood fibrin suspended in bouillon, and Uschinsky's

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7. Herter & Broeck. Jour. Biol. Chem. Vol. 9, p. 491.
8. Nawiasky. Arch. für Hyg. Vol. 64, Part 1, p. 33.
9. Rettger. Jour. Biol. Chem. Vol. 2, p. 71.
10. Rettger & Newell. Jour. Biol. Chem. Vol. 13, p. 341.

medium and concluded that, while actively proteolytic, B. proteus does not form those end products characteristic of putrefaction in the narrow sense of the word. Effront(11) studied B. proteus, B. sporogenes, B. mesentericus, B. butyricus, B. putrificus Bienstock, etc., in a similar manner on albuminoid media. Sasaki's (12,13) researches on the particular substances formed from glycyl-glycine, glygyl tyrosine, cystin and tyrosin are of interest and value as pictures of a certain stage of protein degradation, as are the works of Brasch (14) on glutamic acid, serin, and tyrosin.

Another course by which the study of proteolysis may be pursued is the isolation of enzymes from cultures, usually by filtration, and the investigation of their specificity as well as the comparative amounts present at various periods of incubation. Fermi(15) made elaborate researches of the chemical and physical properties of the enzymes secreted by bacteria. Other investigators such as Groër (16), Jordan(17), Berthiau(18), and Malfitans and Strada(19) confined themselves to the subject of gelatinases.

While investigations like those mentioned are of diagnostic importance, and throw much light upon the types of chemical activity of which a given bacterium is capable, a definite idea of the metabolism of an organism cannot be obtained from purely qualitative

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11. Effront. Monit. Scient. Quesneville. Vol. 1, p. 489.
12. Sasaki. Biochem. Zeitschr. Vol. 41, p. 174; Vol. 47, pp.462, 472; Vol. 59, p. 429.
13. Sasaki, Takaoki & Otsuka. Biochem. Zeitschr.Vol.39, p. 208.
14. Brasch. Biochem. Zeitschr. Vol.18,p.380; Vol. 22, p.403.
15. Fermi. Centralblatt. f. Bakt. u. Par. Vol. 10, p. 401; Vol.68, Orig. p. 433; Vol. 69, Orig. p. 465.
Archiv f. Hyg. Vol. 10, p. 1; Vol. 14, p. 1.
16. Groër. Biochem. Zeitschr. Vol. 38, p. 252.
17. Jordan, E. O. Biol. studies by pupils of W. R. Sedgwick, Boston, 1906, p. 124.
18. Berthiau. Centralblatt f. Bakt. u. Par., Orig. Vol.74,p.374.
19. Malfitans & Strada. C. r. Soc. de Biol. Vol. 59, p. 195.

data or from a single quantitative determination of some particular product. For such purposes a series of quantitative determinations should be made on a favorable medium, under conditions of vigorous normal growth. Many factors enter into the nature and amount of decomposition products formed:- first, the availability of the nitrogenous compounds present, depending upon whether or not the organism in question is capable of elaborating a ferment to digest them, the molecular point of attack of the enzyme, whether intermediate products are present, and, if so, what and in what quantity, what proportion of the split molecules are resynthesized into bacterial protein and which are left in the media, and whether or not some of the compounds present are assimilated in preference to others. In the case of any particular end product it is desirable to know whether it represents a portion of the protein molecule unattacked by the bacteria, a true cell excretion, or something set free by the death of the cell or by autolysis. Any systematic investigation of the proteolytic activity of an organism should include at least the following points:

1. Determination of the amounts present of certain available food substances.
2. Periodic determination of the amounts of one or more decomposition products used as a measure of proteolysis.
3. Allowances for such factors as rate of increase in numbers, presence of other non-nitrogenous food stuffs, presence of decomposition products toxic to the cells or inhibitory of their activity, and products of autolysis of dead cells.

The infinite complexity of proteins, their delicate susceptibility to change under the influence of chemical and physical agents used

for their isolation and purification, and the purely technical difficulties involved in an attempt to avoid accidental contaminations while preserving optimum conditions of growth as well as the refinements of chemical technique necessary to quantitative determination, have tended to discourage this type of research, with the result that there are comparatively few such studies on record, all more or less incomplete.

Among the end products of putrefaction which have been determined quantitatively as a measure of proteolysis, ammonia has been the most common. Effront(20) made use of it in his studies on Yeast. Emmerling and Reiser(21) stated that at least 25% of the nitrogen in gelatine was converted into ammonia by B. fluorescens liquefaciens, while Arnaud and Charrin(22) found that in three days B. pyocyaneus converted 91% of the nitrogen in asparagin into ammonia and that a three week's culture in gelatine contained ammonia to the amount of 70% of the total nitrogen. Boencke(23) and Kendall and Farmer(24) determined ammonia to check the degree of proteolysis in their studies on the protein-sparing influence of carbohydrate. While the information thus obtained is valuable, particularly in the case of the putrefactive saprophytes, the ammonia curve admits of varying interpretations unless checked by other determinations. Moreover, many pathogens do not liberate much ammonia during their short period of life on artificial media (Berghaus,25). The quantitative determination of indol (26,27,28) and of phenol(29, 30), val-

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20. Effront. C. r. Acad. de Sciences, Vol. 146, p. 779.
21. Emmerling & Reiser. Ber.d. deutsch. chem. gesellsch. 1902, part 3, p. 700.
22. Arnaud & Charrin. C. f. Acad. de Sciences, Vol.102, p.755.
23. Boencke. Arch. f. Hyg. Vol. 74, p. 81.
24. Kendall & Farmer. Jour. Biol. Chem. Vol.12,p13; Vol.13,p.63.
25. Berghaus. Arch. f. Hyg. Vol. 64, part 1, p. 1.
26. Monotte & Demanche. C. r. Soc. de Biol. Vol. 64, p. 658.

27. Porcher & Panisset. C. r. Soc. de Biol. Vol. 70, p. 438.
28. Seidelin. Jour. of Hygiene, Vol. 11, pp. 118 and 503.
29. Debrowolski. Annales..Inst. Pasteur. vol. 24, 594.
30. de Giacomo. C. r. Soc. de Biol., Vol 67, p. 720.

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uable as they are in the study of B. coli, and others of the intestinal flora and similar organisms, has the same limitation.

At the other extreme of the metabolic process, the progress of proteolysis may be observed by systematic determinations of the amount of coagulable protein remaining in the culture medium. De Waele and Vendevelde(31) worked out these results with a variety of organisms planted on gelatine, milk, and casein bouillon in an endeavor to settle the question of the specificity of bacterial proteases. Bainbridge(32), Rettger(33,34,35) and Sperry(35), working with representative pathogenic and saprophytic strains on media composed of mineral salts and pure protein (crystallized egg albumin, serum protein, alkali albumen, and edestin) have proven that such growth as may occur on solutions of unchanged proteins is due to the minute amounts of nitrogenous impurities present, and that in the absence of split-protein products the germs are unable to attack native albumen.

The quantitative determination of amino-acids is particularly useful, especially if checked by comparison with the amount of destruction of the protein or split-protein which is their source, or, in experiments using the amino-acids as a source of nitrogenous food, estimations of the ammonia which is their fate (Nawiasky 36). Until very recently Sorensen's formol-titration meth-

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31. de Waele & Vendevelde. Centralblatt f. Bakt. u. Par., Orig. Vol. 39, p. 353.
32. Bainbridge. Jour. of Hygiene, Vol. 11, p. 341.
33. Rettger. Am. Jour. Physiol. Vol. 8, p. 284.
34. Sperry. Report at Soc. of Am. Bact., Montreal, Dec. 31, 1913.
35. Sperry & Rettger. Jour. Biol. Chem. Vol. 20, p. 445.
36. Nawiasky. Arch. f. Hygiene, Vol. 66, part 3, p. 209.

od(37) has been the most in use. It depends upon the fact that formalin unites with free amino- groups to form methylene addition compounds, but does not react with the the imino (-NH-) groups. The sample is treated with 40% formalin solution neutralized to phenolphthalein with sodium hydroxid and the reaction resulting (which may be represented by the equation

$$R - CH.NH_2 - COOH + CH_2O \rightarrow R - CH - N = CH_2 - COOH + H_2O)$$

reduces the alkalinity of the NH_2 group, so that the increased acidity may be titrated with standard sodium hydroxid and the amount of nitrogen as amino-acid calculated. This procedure was followed by Rosenthal and Patai(38) who planted virulent and avirulent strains of Staphylococcus, Streptococcus and Bacillus coli on broth. With all three of these organisms they observed an initial sharp rise of amino-acid, followed by a more gradual rate of increase. Meserintsky(39) observed a progressive augmentation of the amount of amino-acid present in a 10% gelatine culture of B. prodigiosus, which, however, represented only a portion of the non-coagulable nitrogenous material present. Frouin and Ledebt(40), working with B. coli, B. typhosus, B. dysenteriae and V. cholerae on a solution of non-nitrogenous mineral salts enriched with amino-acids were unable to detect any decrease during the first twenty-four hours of growth, though the diminution became apparent later. Kendall, Day and Walker (41) attempted to use the Sorensen technique in addition to determinations of ammonia, total nitrogen and acidity in their work on

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37. Sorensen. Biochem. Zeitschr. Vol. 7, pp. 45 and 407.
38. Rosenthal & Patai. Centralblatt f. Bakt.u. Par.,Orig.Vol.73, p. 406.
39. Meserintsky. Biochem. Zeitschr. Vol. 29, p. 104.
40. Frouin & Ledebt. C. r. Soc. de Biol. Vol. 70, p. 24.
41. Kendall, Day & Walker. Jour. Am. Chem. Soc. Vol. 35, p. 1201.

the protein-sparing action of glucose, but abandoned the amino-acid estimation because "the results furnished no information of importance."

The more delicate and accurate method of Van Slyke(42) now makes it possible to obtain much more significant estimations of the progress of proteolysis, since the micro-apparatus is accurate to .005 milligrams of nitrogen. Under the influence of glacial acetic acid and sodium nitrite the amino- group is broken, liberating free nitrogen. The oxides of nitrogen which are set free from the reagents, as well as any carbon dioxide, are absorbed by shaking with alkaline potassium permanganate, and the remaining pure nitrogen is measured in a gas burette. One half the nitrogen thus determined represents the amount originally present in the NH_2 group.

$$R - CHNH_2 - COOH + HNO_2 \rightarrow R - CHOH - COOH + 2N + H_2O.$$

Van Slyke found that the alpha amino-acids react quantitatively within five minutes at 20°, while ammonia and methylamine require one and one-half to two hours, and urea eight. The nitrogen bound in the - CO - NH - linking does not react, as evidenced by the fact that glycyl anhydride gives off no nitrogen.

This method was used by Sears(43) on peptone, broth, and gelatine cultures of a great variety of organisms. He also determined ammonia and creatinin. While some of his cultures showed a gradual increase in amino-nitrogen, the majority exhibited fluctuating rises and falls indicating in his opinion that amino-acids were formed and broken continuously during the life of the culture. The protein-sparing influence of glucose was made evident by the amino-

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42. Van Slyke. Jour. Biol. Chem. Vol.9, p.185; Vol. 12, p. 275; Vol. 16, p. 121; Vol. 23, p. 407.
43. Sears. Jour. Inf. Dis., Vol. 19, p. 105.

acid curve in two instances (B. fecalis alkaligenes and B. dysenteriae Shiga) where the ammonia curve did not show it.

To arrive at a more nearly complete understanding of the nitrogen metabolism of bacteria it would seem valuable not only to apply such a series of tests to growth on peptone-containing media, but also to study the amino-acid content of cultures on some material which contains native albumen. This is particularly true of the pathogens, many of which do not thrive on plain peptone or broth. Moreover, their behavior in a medium closely resembling blood-serum or some other body fluid is well worth detailed consideration, both for the insight it might give into the process of infection, and the possibility of the usefulness of such data in attacking the chemical side of problems of immunity. Ascitic fluid is more similar to blood serum than any other material which can easily be obtained sterile in sufficient quantity for such purposes. Accordingly, determinations have been made on B. typhosus, B. coli, B. proteus, B. pyocyaneus, Staphylococcus, and Meningococcus grown on broth, on pure ascitic fluid, and on mixtures of equal quantities of the two. In order to rule out the protein-sparing properties of carbohydrates as much as possible in the interests of simplicity, sugar free broth was used. Meat infusion was freed of muscle sugar by fermentation with B. coli. To the extract from 500 grams of beef was added 10 grams of peptone and 5 grams of salt, the whole made up to a liter, adjusted to 1% normal acidity, boiled, autoclaved, etc., in the usual manner. Such broth may contain considerable amounts of amino-acid determined by Van Slyke's method, but no coagulable albumen or carbohydrates. A typical flask affords .138 mgr. of amino-acid nitrogen per cubic centimeter, while the amount of nitrogen present

as coagulable protein was within the limits of experimental error (.003435 mgr. per cubic centimeter). Other samples show as much as .35 mgr. of amino-acid per cubic centimeter. In ascitic fluid, on the other hand, the amount of amino-acid is small, while the nitrogen present as coagulable protein is considerable (nearly 3 mgr. per cubic centimeter in one instance). A small amount of carbohydrate is probably present but no tests for it were made. A mixture of equal parts of broth and ascitic fluid represents a middle ground between these conditions. In the experiments recorded in Tables V and VI ascitic fluid was distributed in equal amounts (10 c.c.) in a series of sterile test tubes, and each inoculated with a loopful of broth culture. At regular intervals the amino-acid content of one tube was determined, the loss by evaporation being carefully made up with distilled water. While this method has the advantage of permitting determinations to be made without danger of contamination of the material destined for later analysis, there is always the possibility of a considerable variation in the seeding of the different tubes or even in the samples of ascitic fluid. The rest of the work was done on flasks containing a quarter of a liter or more of the medium. Tight fitting rubber caps over the cotton stoppers protected against evaporation. At intervals samples were removed with sterile pipettes after shaking the flask to render the medium homogeneous. These were centrifuged and the supernatant liquid (practically, though probably not absolutely, free of bacterial cells) was tested for the amount of amino-acid present, and in most cases for coagulable protein. In cases where numbers of bacteria present are given, they were determined by counting agar plates made with the customary quantitative precautions. The Kjeldahl determinations were run in

duplicate, and the results averaged if they did not check but showed a difference of less than .5 c.c. in the titration. Figures showing a wide variation were discarded. Two Van Slyke determinations were made routinely, checked by a third, in case the amounts of nitrogen liberated did not agree within .03 c.c.

To show the effect of varying amounts and kinds of amino-acids, .1 c.c. of 48 hour broth culture of B. typhosus was inoculated into each of five flasks of broth which differed only in that Flask I was ordinary sugar free broth containing 1% peptone, Flask II contained in addition .05% tyrosine, Flask III .2% asparagin, Flask IV .5% glycocol, and Flask V extra peptone making a strength of 3%. The results are given in Table I and represented graphically in Chart I.

Table I. B. typhosus.

Mgr. of N. as amino-acid per c.c. on broths of varying amino-acid content

Incubation	Flask I 1% peptone	Flask II .05% tyrosine 1% peptone	Flask III .2% asparagin 1% peptone	Flask IV .5% glycocol 1% peptone	Flask V 3% peptone
Sterile	.357	.401	.520	.715	.678
16 hrs.		.343			
18 hrs.				.686	
36 hrs.	.321	.295			
38 hrs.			.452	.651	.667
60 hrs.	.277	.282		.605	
90 hrs.			.272	.577	.806
10 days	.309	.315		.619	.663
18 days	.277	.265	.389	.567	.686

In Flask I we note a gradual decrease in amino-acid during the first two days. This period covers the lag-phase(44) and the period of most rapid multiplication, during which it uses those acids present in the media which the organism is especially able to assimilate. The fact that no great change was apparent at the times when subsequent tests were made would seem to indicate that the peptone in the culture was being used, and that the resulting decomposition products were either synthesized into bacterial protoplasm or else carried beyond the amino-acid stage. However, there seems to be a certain amount of amino-acid present which remains untouched, doubtless being unsuitable as nutriment for the cells. In the tyrosine flask (II) the same level is reached and maintained after 60 hours, indicating that the extra food stuff in that form was readily assimilated. Flask III (with asparagin) shows the same process in a more striking degree. It is unfortunate that no 60 hour test was made to determine whether the asparagin was used up in that length of time, or really required three days and a half to reach the dead level as the chart seems to show. Flask IV, which contained a considerable amount of glycocol, shows a similar absolute decrease, but the level maintained is higher than that of the other flasks by just about the amount of glycocol added. (The difference in amino-acid nitrogen between Flasks I and IV was .358 milligrams when sterile, and .354 milligrams after 10 days incubation). This apparently indicates that glycocol is not assimilated by B. typhosus when other sources

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44. Lane-Claypon. Jour. of Hygiene, Vol. 9, p. 239.

of nitrogen are present. It would be interesting to know whether other organisms which do not liquefy gelatine possess the same peculiarity. Aurel and Colin (45) found that B. pyocyaneus (a liquefier) could live on a medium of inorganic salts, citric acid, and glycocol but formed no pyocyamin. No report of research as to the behavior of B. typhosus with glycocol as the sole source of nitrogen has come to hand, although Sasaki (46) states that it can break down glycylglycine. On Flask V there is very little decrease during the first day and a half, indicating that the free amino-acid used up is balanced by the fact that some is being set free from the peptone. After this, apparently the peptone destruction sets free more acid than is used up, but later still these are synthesized or more likely broken down into simpler substances. (Sears(47) found that B. typhosus formed considerable amounts of ammonia on peptone solution.)

The results obtained with the same strain on a mixture of sugar free broth and ascitic fluid (Table II and Chart II) are of interest in connection with the first experiment:

Table II. B. typhosus

Mgr. of N per c.c. as amino-acid and as coagulable protein in a mixture of broth and ascitic fluid.

Incubation	Amino-acid	Coagulable protein	Bacteria per c.c.
Sterile	.430	.859	
12 hrs.	.439	.818	384,000,000
36 hrs.	.354	.805	
60 hrs.	.551	.639	1,580,000,000
84 hrs.	.581	.488	224,000,000
10 days	.385	lost	102,000,000

45. Aurel and Colin. C. r. Soc. de Biol. Vol. 74, p. 790.
46. Sasaki. Biochem. Zeitschr. Vol. 47, p. 462.
47. Sears. Op. cit. p. 117.

Here the protein is attacked from the first, and there is even a slight rise in amino-acid during the lag-phase followed by a rapid rise in free amino-acid, and great destruction of protein continuing even a little after the maximum count was reached. Then ensues a period during which the amino-acids are gradually used up by the germs. Apparently the amino-acids set free by autolysis of dead germs is more than accounted for by the enzymes present.

The results obtained on two flasks of broth inoculated with B. coli (Table III and Chart III) are comparable to those with B. typhosus:

Table III. B. coli.

Mgr. of N as amino-acid per c.c. in broth.

Incubation	Flask I	Flask II
Sterile	.329	.329
1 day	.239	.227
2 days	.224	.308
4 days	.153	.175
8 days	.235	.226

The two flasks of broth inoculated with B. proteus (Table IV and Chart IV) indicate that, after the first drop due to the utilization of the amino-acids present, very little of the amino-acid broken from the peptone is left free in the medium, and the accumulation is not great.

Table IV. B. proteus

Mgr. of N as amino-acid per c.c. of broth

Incubation	Flask I	Flask II
Sterile	.329	.329
1 day	.239	.233
2 days	.191	.202
6 days	.230	.235
8 days	.282	.282

On undiluted ascitic fluid (Table V and Chart V) this is even more marked.

Table V. B. proteus.

Mgr. of N as amino-acid per c.c. of pure ascitic fluid								
Incubation	Sterile	1 da	2 da	3 da	4 da	5 da	6 da	7 da	12 da
Mgr. N.	.077	.060	.054	.043	.026	.031	.054	.032	.026

Unfortunately determinations of coagulable protein were not made, but the idea that the small amount of free amino-acid is due to thoroughness of decomposition rather than lack of protein gains strength when one observes the amounts of amino-acid set free from the same fluid by B. pyocyaneus (Table VI and Chart VI).

Table VI. B. pyocyaneus.

Mgr. of N as amino-acid per c.c. of ascitic fluid							
Incubation	Sterile	1 da	2 da	3 da	5 da	8 da	10 da	12 da
Mgr. N.	.077	.049	.013	.074	.099	.249	.493	.695

On sugar free broth B. pyocyaneus shows a variation (Table VII and Chart VII) which may be significant. After the original drop (which would perhaps be more noticeable if a two-day determination had been made) and a subsequent increase between the third and sixth days, there is an unmistakable drop shown on the eleventh day. Can it be that after exhausting the favorite amino-acids present in the peptone, the organism attacks the previously unused amino-acid while in an abundance of available protein it continues uneconomically to select for synthesis the molecules best adapted to its needs?

Table VII. B. pyocyaneus.

Mgr. of N as amino-acid per c.c. of broth						
Incubation	Sterile	1 day	3 days	4 days	6 days	11 days
Mgr. N	.1384	.098	.086	.184	.215	.066

On a mixture of broth and ascitic fluid (Table VIII and Chart VIII) the results are somewhat similar, except that the rise in amino-acid occurred earlier. Here, too, we have a decrease in amino-nitrogen after the fourth day, although there is destruction of protein going on at the same time. Curiously enough, the greatest destruction of protein took place after the maximum count had been passed and at a time when the free amino-acids were not noticeable increasing. The protein destruction might be attributed to the activity of the enzymes previously secreted by the bacteria but in that case an increase of amino-acid would be expected. The fact that the acids do not remain free in the culture can be explained on either of two suppositions: that the enzymes secreted were of more than one kind and could carry proteolysis beyond the amino-acid stage or, on the other hand, that division and consequently synthesis of amino-acid into bacterial protoplasm was proceeding at a maximum rate during this period, the apparent decrease in numbers being due to the fact that the death rate began to make itself felt at the point when the count was 2,000,000,000.

Table VIII. B. pyocyaneus.

Mgr. per c.c. as amino-acid and as coagulable protein on ascitic fluid + sugar free broth.			
Incubation	Amino-acid	Coagulable protein	Bacteria per c.c.
Sterile	.441	.646	
12 hrs.	.439	.646	80,000,000
36 hrs.	.520	.616	2,000,000,000
60 hrs.		.529	560,000,000
84 hrs.	.537	.433	206,000,000
10 days	.386	.172	10,000,000

The Staphylococcus cultures on broth (Table IX and Chart IX) show a drop to .28 mgr. though Flask I originally contained nearly half again as much amino-acid as Flask II. Flask I on the chart is shown as having reached this minimum two days later than Flask II but this may well be due to the fact that no three day determination was recorded. In Flask II there was a steady gradual rise after reaching the minimum until the point when it had to be discarded on account of contamination. Flask I shows fluctuations which are without significance when not checked by determinations of other substances.

Table IX. Staphylococcus

Mgr.N as amino-acid per c.c. in broth.		
Incubation	Flask I (2% peptone)	Flask II (1.5% peptone)
Sterile	.606	.407
1 day	.438	.333
2 days		.315
3 days		.280
5 days	.280	.319
7 days	.390	.328
9 days	.332	.376
11 days		.369
14 days	.252	
19 days	.380	

On a mixture of broth and ascitic fluid (Table X and Chart X) the amino-acid curve shows the usual decided fall during the period of most rapid multiplication, followed by a rise and later some fluctuation. The coagulable protein curve is not so satisfactory. This may be due to the fact that the ascitic fluid used in the ex-

periment, although sterile, showed some tendency to form a stringy coagulum when mixed with broth, and in spite of vigorous shaking, the samples pipetted out may not always have been representative. That is the only reasonable explanation of the apparent rises between the 60 and 84 hour points and again between the last two determinations.

Table X. Staphylococcus.

Mgr. N per c.c. as amino-acid and as coagulable protein in a mixture of ascitic fluid and broth.

Incubation	Amino-acid	Coagulable protein	Bacteria per c.c.
Sterile	.468	1.420	
12 hrs.	.393	1.382	192,000,000
36 hrs.	.295	1.312	300,000,000
60 hrs.	.306	1.148	40,000,000
84 hrs.	.445	1.189	
132 hrs.	.425	1.158	3,000,000
8 1/2 days	.341	1.052	1,400,000
14 days	.390	1.134	

The only instance of a decided rise in amino-acid content during the first twenty-four hours was observed in a culture of Meningococcus on a mixture of broth and ascitic fluid (Table XI and Chart XI). As the coagulable protein was not destroyed during this period, there must have been considerable decomposition of peptone, not all the resulting amino-acids being assimilated with equal avidity.

Table XI. Meningococcus.

Mgr. N per c.c. as amino-acid and as coagulable protein in a mixture of broth and ascitic fluid.

Incubation	Amino-acid	Coagulable protein	Bacteria per c.c.
Sterile	.296	2.633	
12 hrs.	.579	2.640	70,000,000
36 hrs.	.464		350,000,000
84 hrs.	.408	2.550	91,000,000
108 hrs.	.322	2.454	52,000,000

CONCLUSIONS

I. All the organisms studied attack the coagulable proteins present in ascitic fluid.

II. On broth, and on mixtures of broth and ascitic fluid, all but the Meningococcus show an initial decrease in the amount of amino-acid, followed by a gradual rise.

III. By the Van Slyke method determinations of small amounts of amino-acid can be made, and consequently there is now available a more delicate measure of the intermediate stages of protein decomposition than those used by earlier investigators. The results obtained are sufficiently significant to encourage further research along these lines.

Chart II. B. Typhosus on Sugar-free Broths of different Amino Acid Content.

— I. Sugar-free Broth (1% peptone)
— II " " " +.05% Tyrosine
— III " " " +.2% Asparagin
— IV " " " +.5% Glycocol
--- V " " " 3% Peptone

mgr. N as Amino Acids.

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 Days

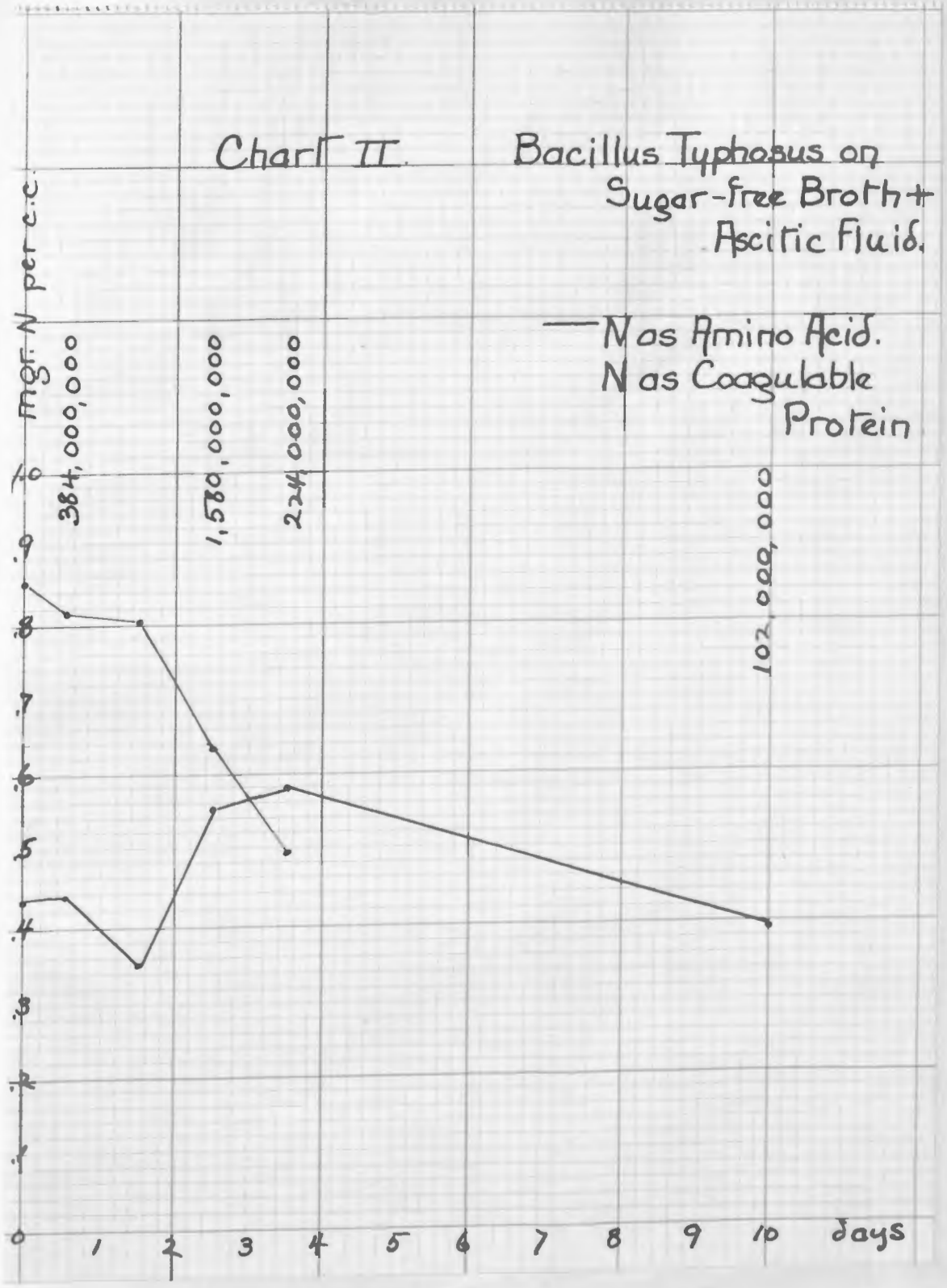


Chart II.
Bacillus Typhosus on Sugar-free Broth + Ascitic Fluid.
— N as Amino Acid.
N as Coagulable Protein
Mgr. N per c.c.
384,000,000
1,580,000,000
244,000,000
102,000,000
0
1
2
3
4
5
6
7
8
9
10
days

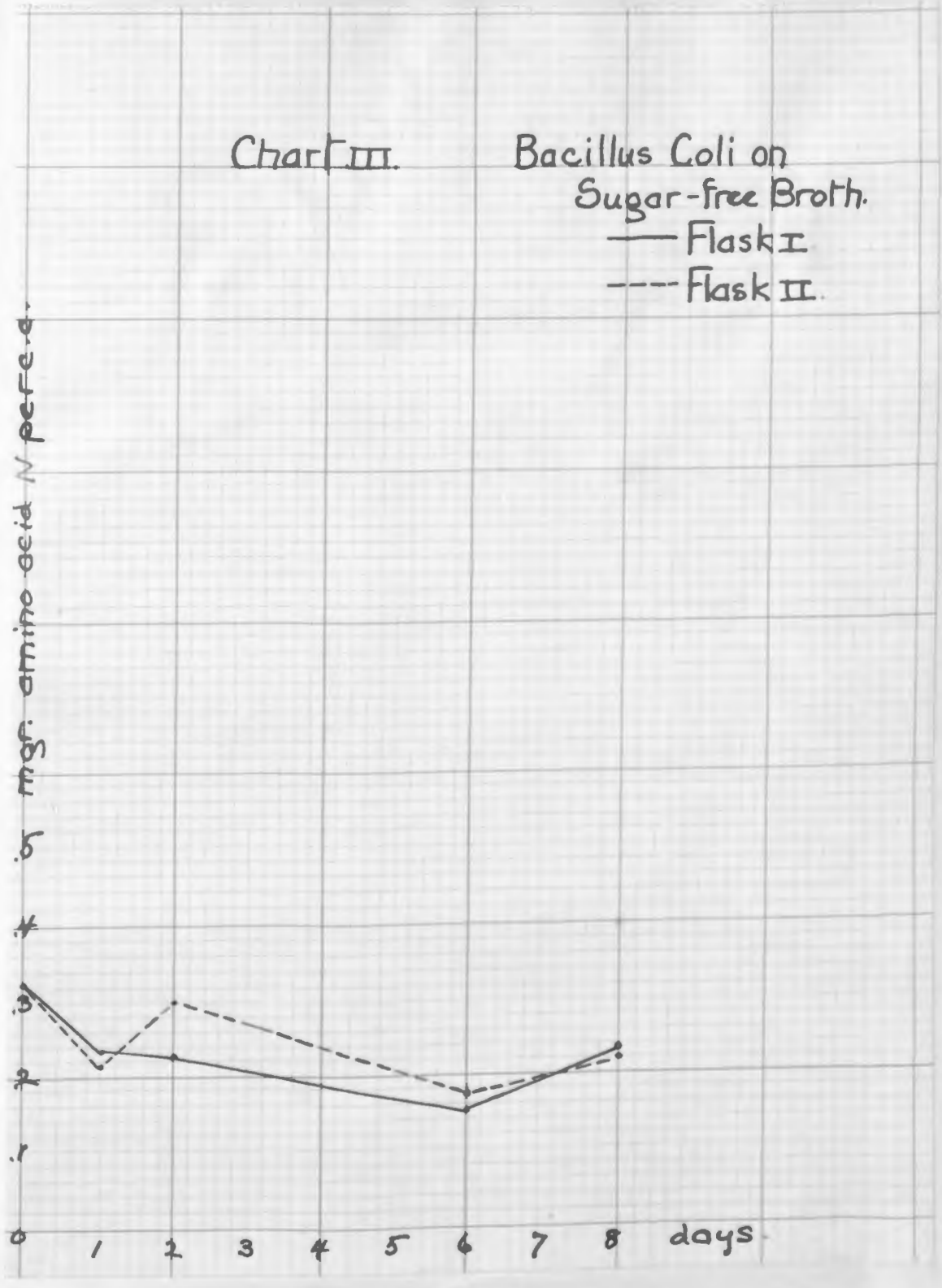


Chart III.
Bacillus Coli on
Sugar-free Broth.
—— Flask I.
---- Flask II.
mgs. amino acid N perc.c.
0
1
2
3
4
5
6
7
8
days

Chart IV.

Bacillus Proteus on Sugar-free Broth.

—— Flask I

--- Flask II.

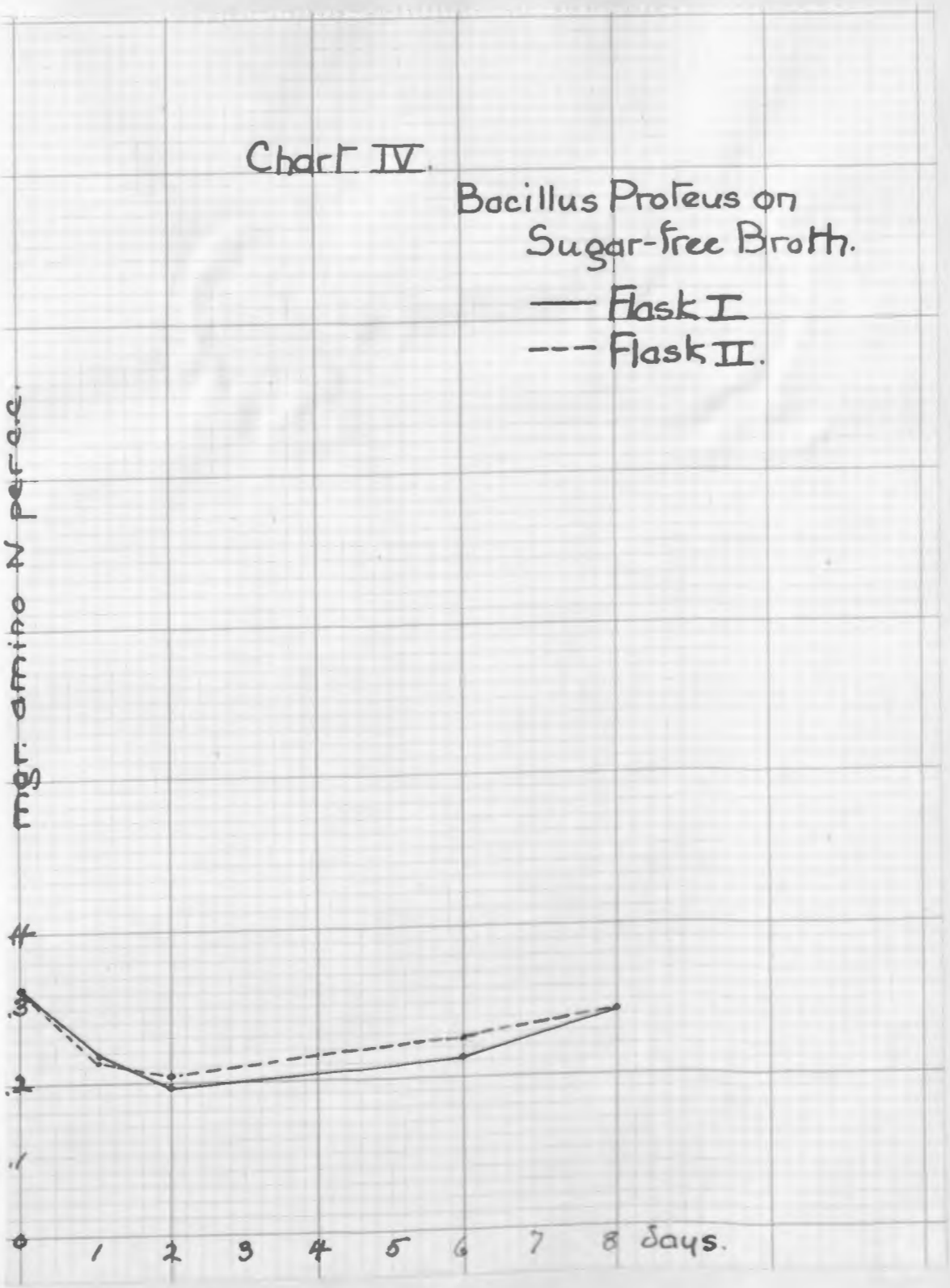


Chart V Bacillus Proteus on Pure Ascitic Fluid.

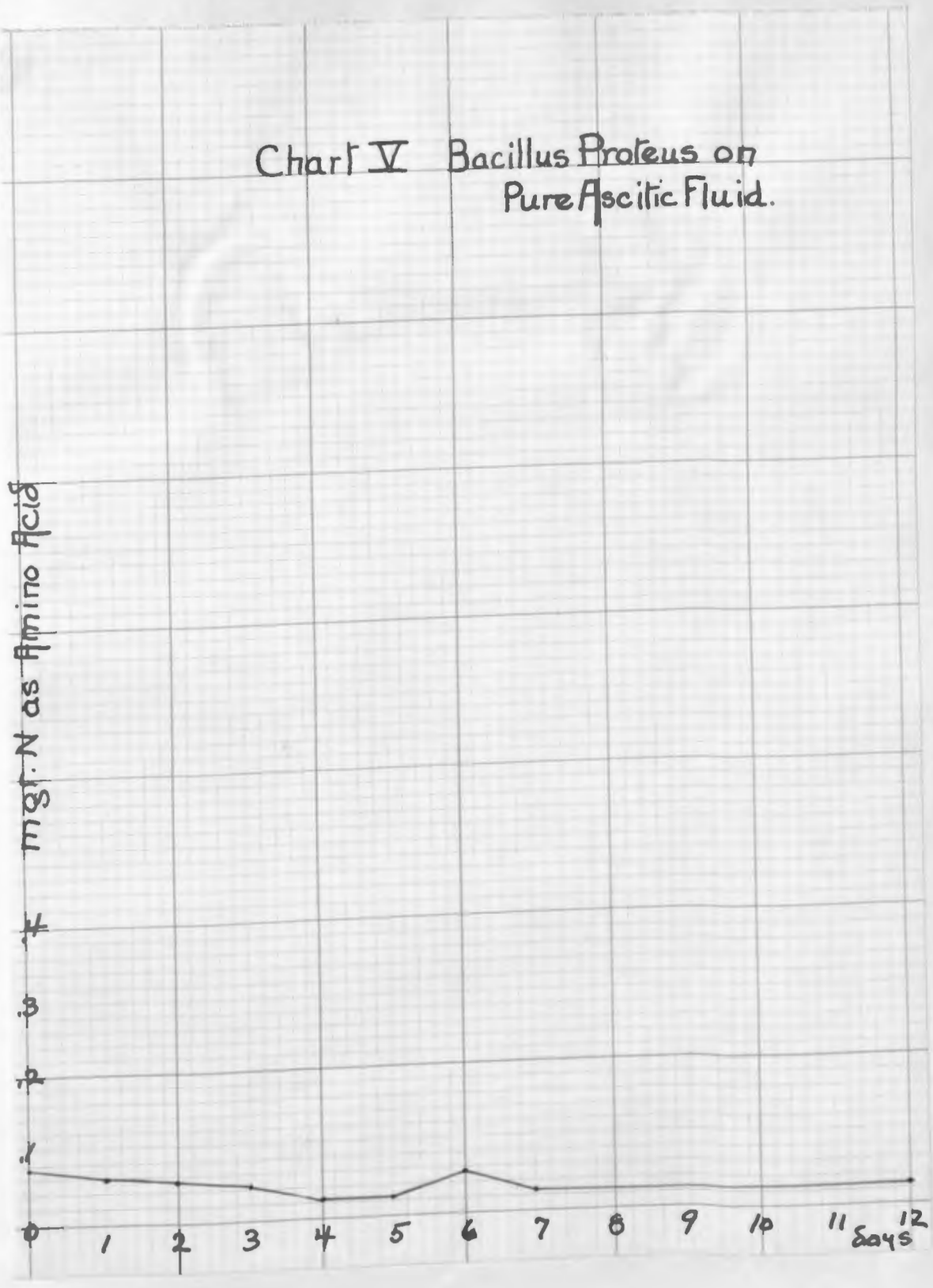


Chart VI. B. pyocyaneus on Ascitic Fluid.

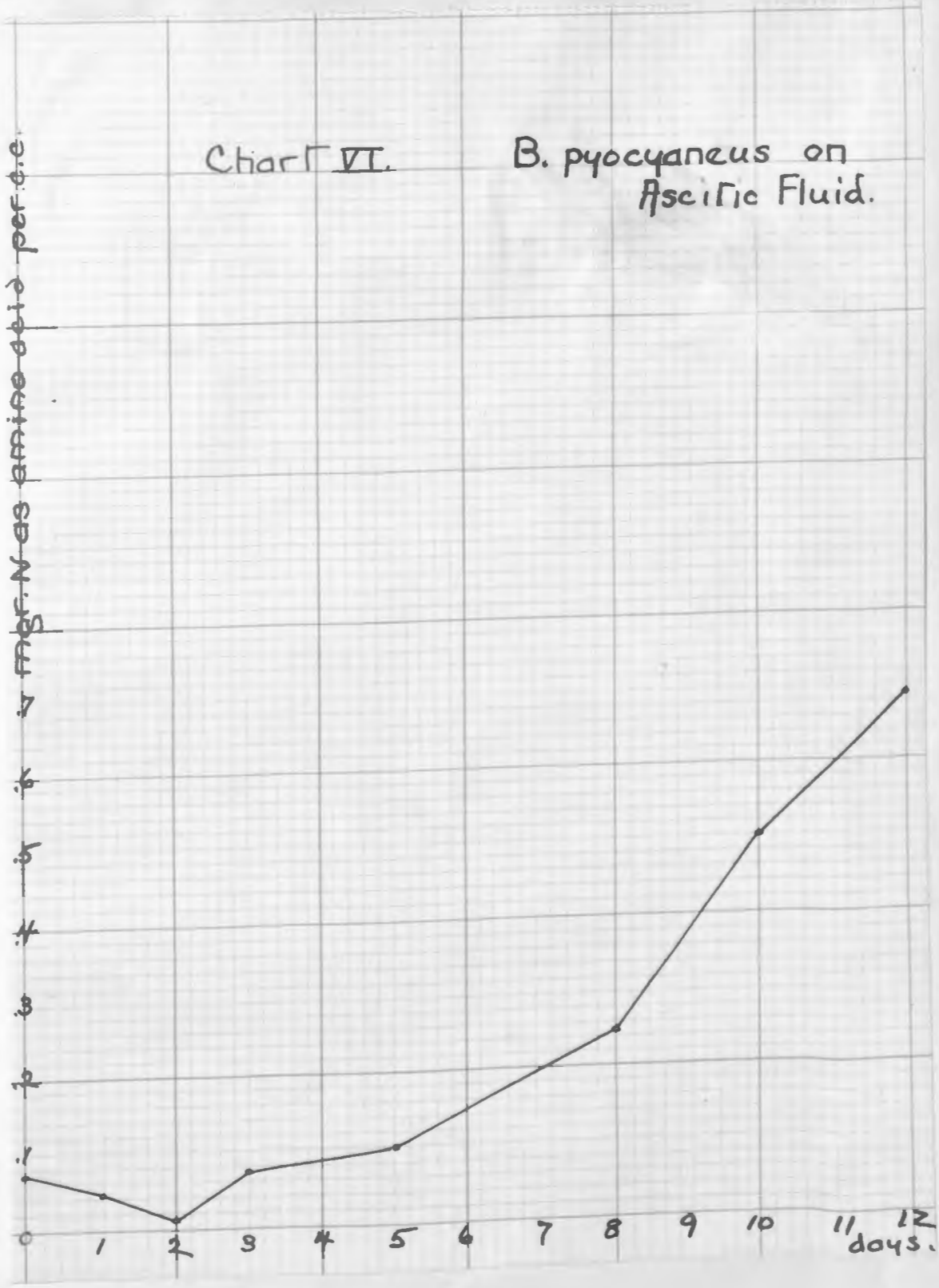
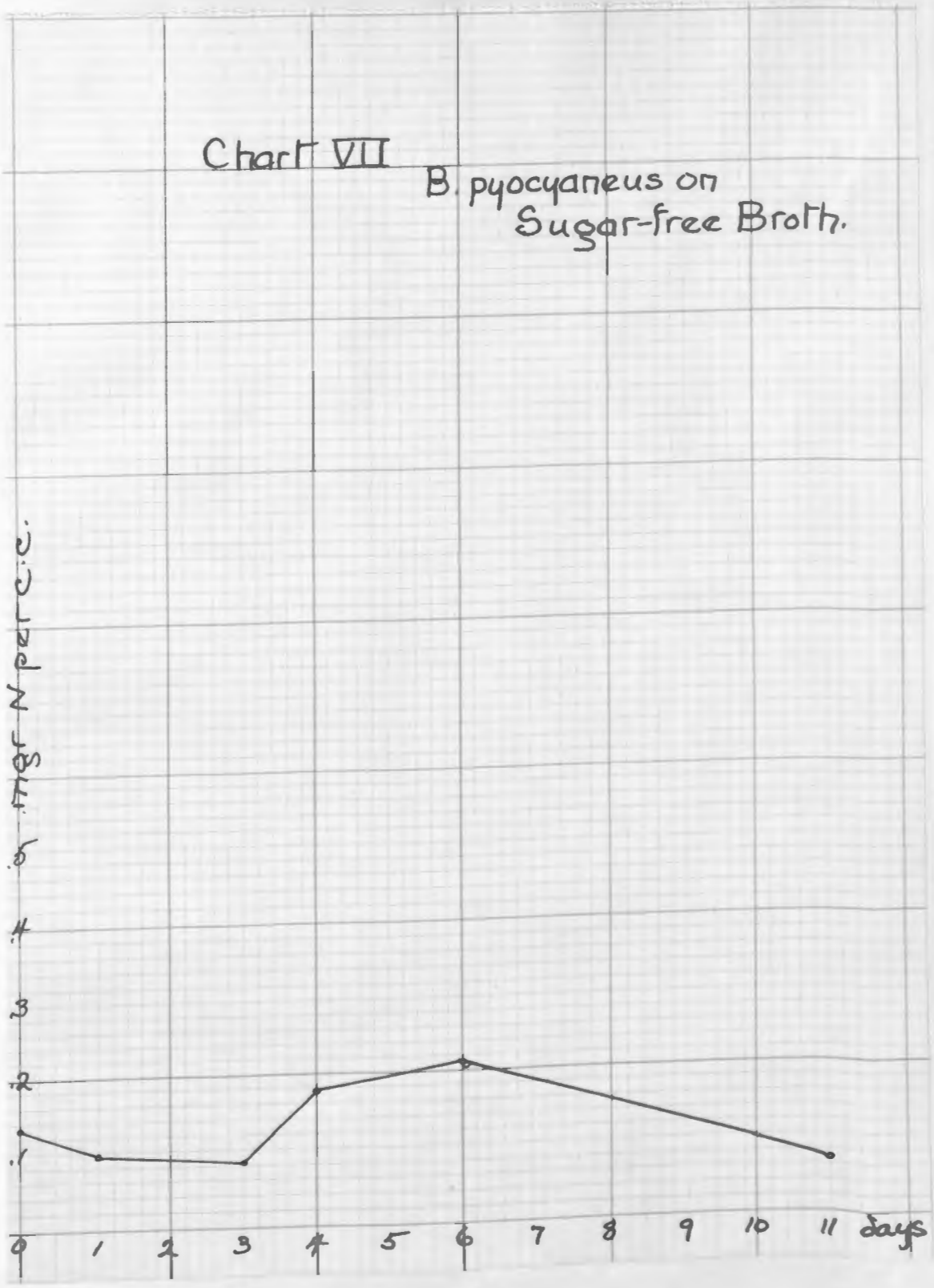


Chart VII

B. pyocyaneus on Sugar-free Broth.



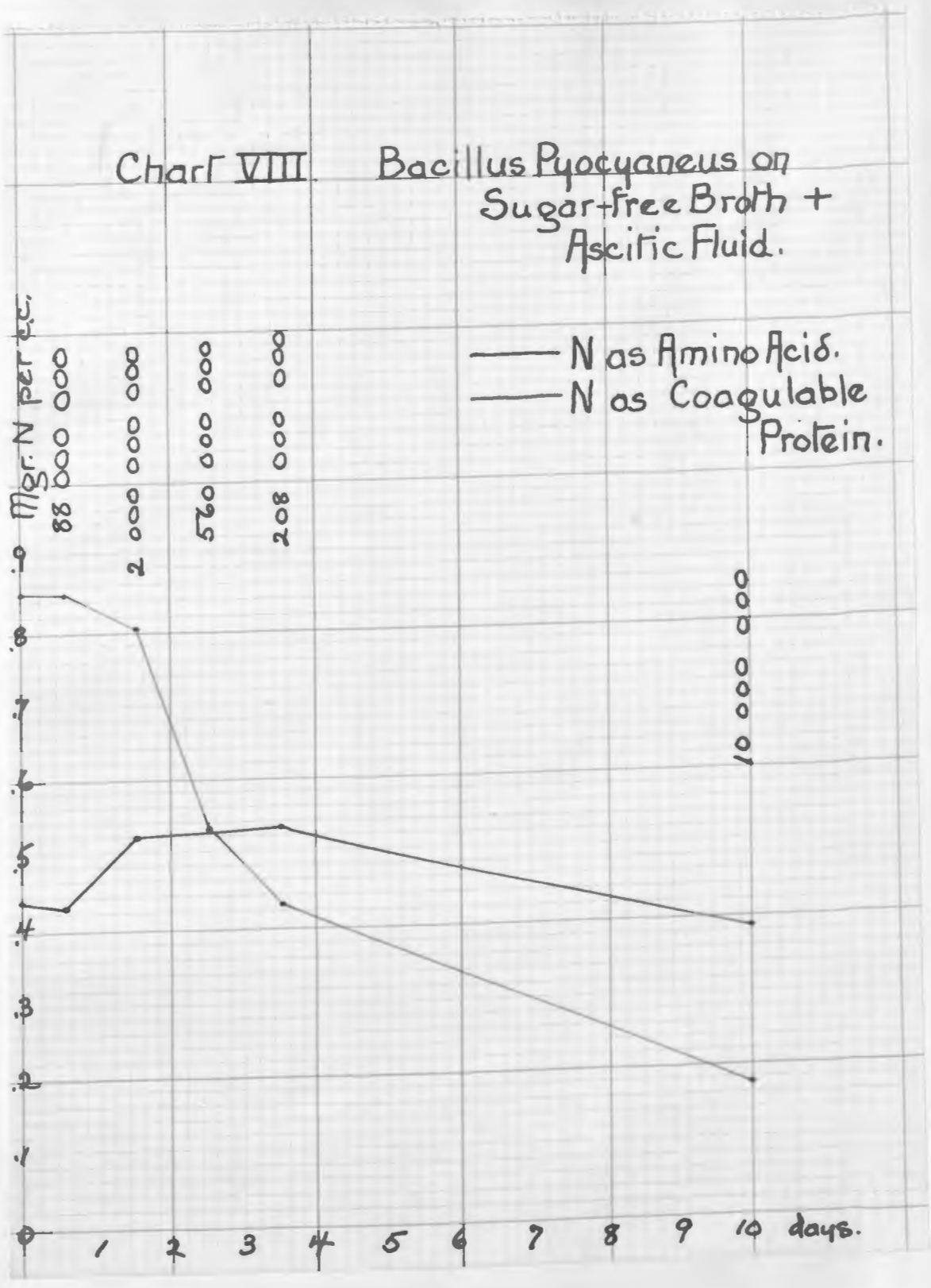
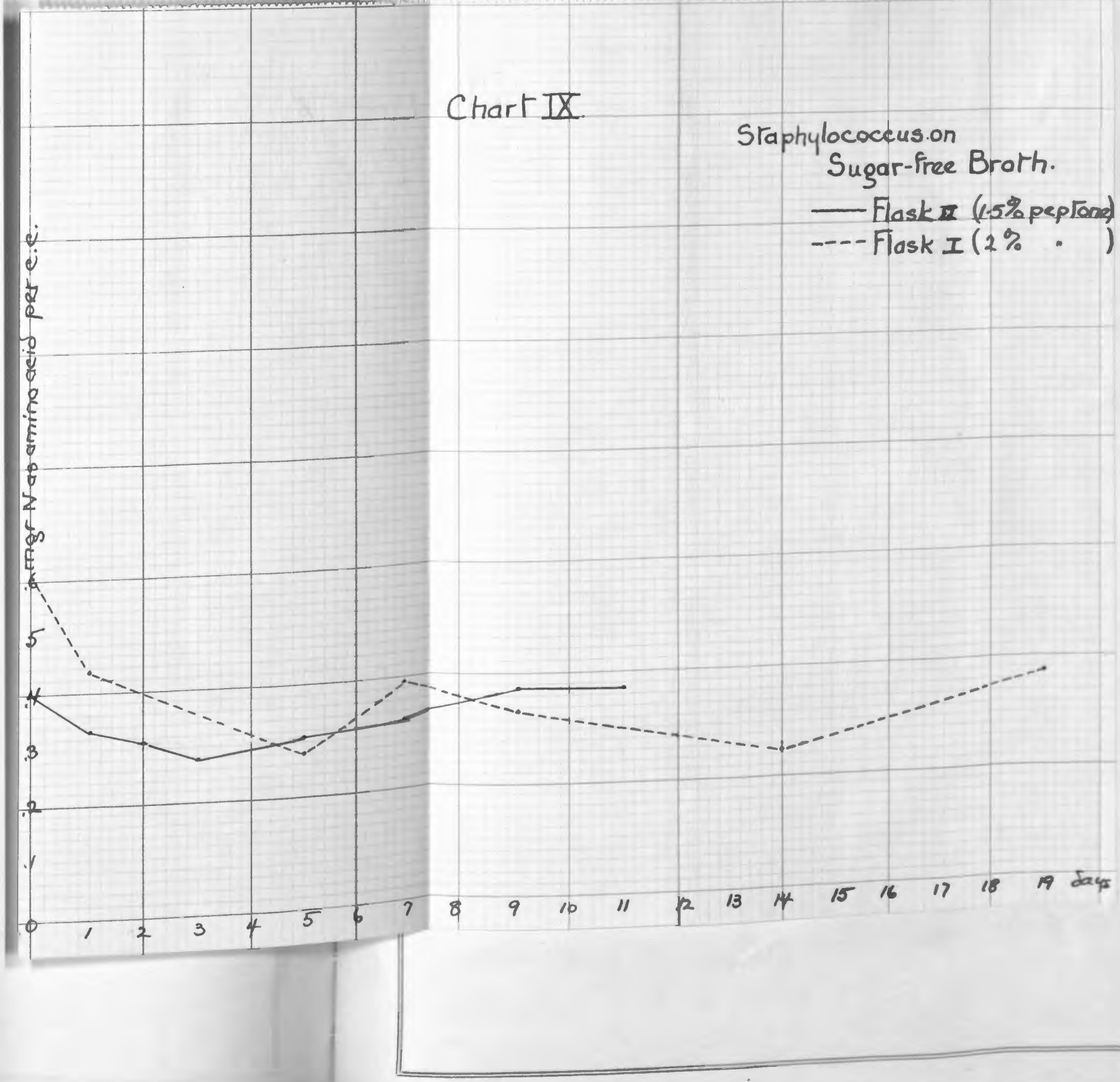


Chart VIII. Bacillus Pyocyaneus on Sugar-free Broth + Ascitic Fluid.
—— N as Amino Acid.
—— N as Coagulable Protein.
Mgr. N per c.c.
88 000 000
2 000 000 000
560 000 000
208 000 000
10 000 000
0
1
2
3
4
5
6
7
8
9
10 days.

Chart IX.

Staphylococcus on
Sugar-free Broth.

—— Flask II (1·5% peptone)
---- Flask I (2% ")



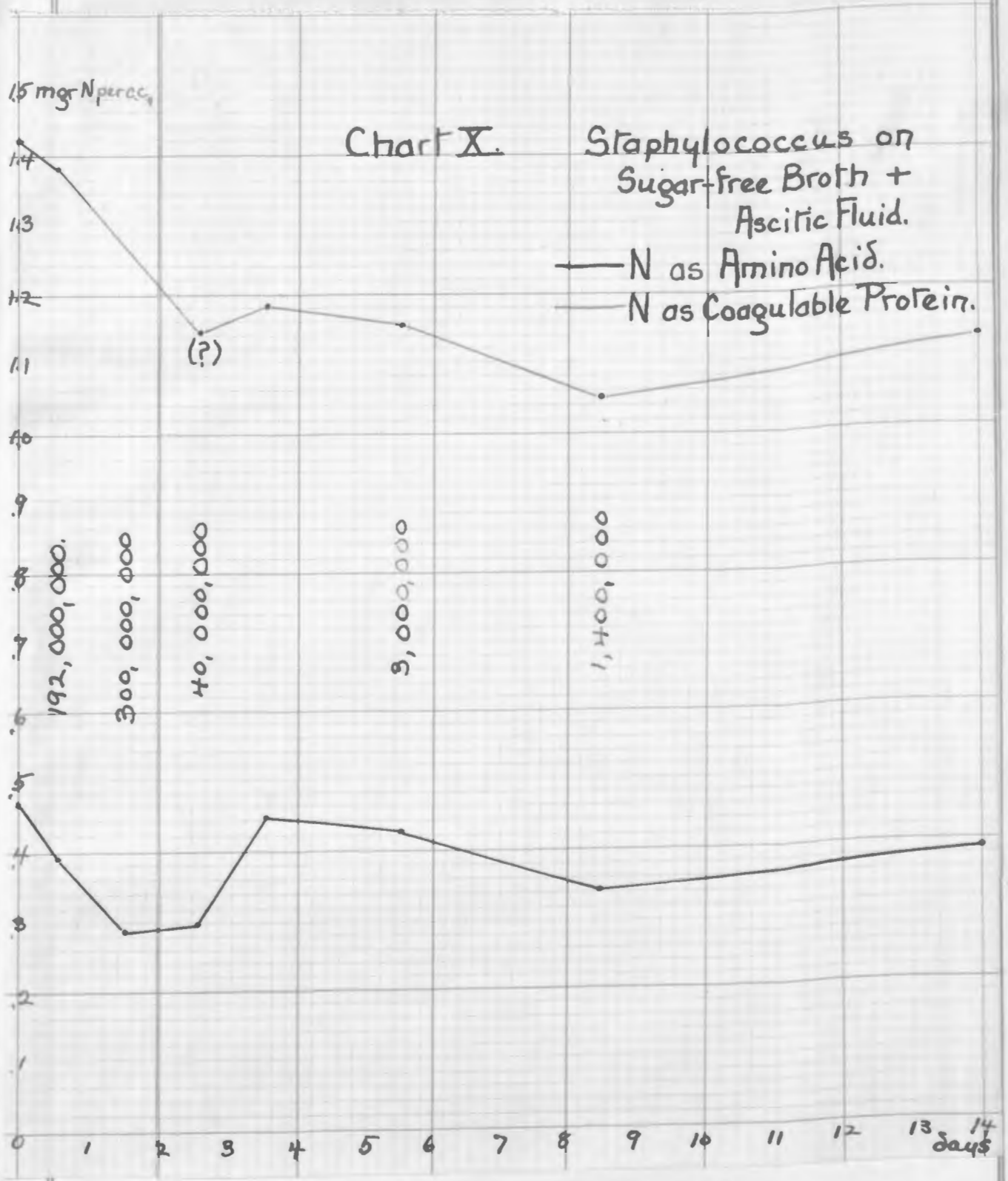
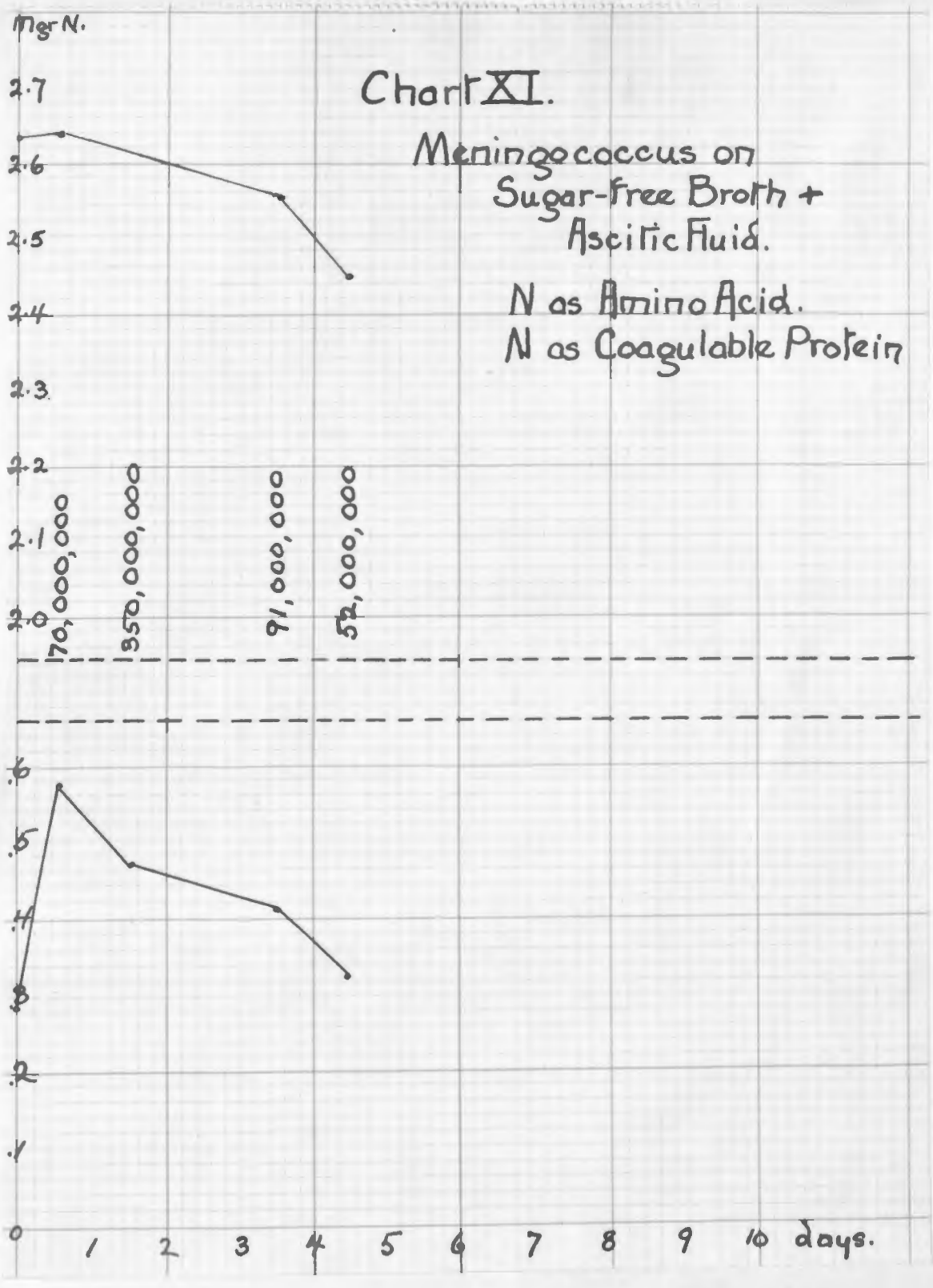


Chart X.
Staphylococcus on Sugar-free Broth + Ascitic Fluid.
N as Amino Acid.
N as Coagulable Protein.
1.5 mgr N per cc.
1.4
1.3
1.2
1.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
192,000,000.
300,000,000
40,000,000
9,000,000
1,400,000
(?)
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Days


Mgr N.
Chart XI.
Meningococcus on
Sugar-Free Broth +
Ascitic Fluid.
N as Amino Acid.
N as Coagulable Protein
2.7
2.6
2.5
2.4
2.3
2.2
2.1
2.0
70,000,000
350,000,000
91,000,000
52,000,000
.6
.5
.4
.3
.2
.1
0
1
2
3
4
5
6
7
8
9
10
days.