# DETERMINATION OF NIACIN SYNTHESIS BY VARIOUS MYCOBACTERIA

BY

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# DETERMINATION OF NIACIN SYNTHESIS BY VARIOUS MYCOBACTERIA

Tuberculosis is a disease which is known to have plagued man for the last 3000-5000 years. Evidences of tuberculosis have been found in the bones of Egytian mummies, and there have been found clinical descriptions of this disease in the writings of the ancient Hindus and Chinese. Almost since the early times, people have considered it a contagious disease, but it had not been proven so until Jean Villemin did so in 1865 by showing that he could transmit the infection by transfering tissue from a tubercular to a healthy animal. In 1882 Robert Koch, a German scientist, isolated the acid-fast bacillus, which he showed to be the causative agent of tuberculosis.[1] For many years following this important discovery, knowledge of the various varieties of mycobacteria was rather limited, but gradually, bacteriologists began to isolate other strains, the most recent of which are the atypical acid-fast bacilli. Atypical bacilli cause considerable difficulty to bacteriologists and clinicians concerned with the treatment of tuberculosis.

Atypical acid-fast organisms are often found in the smears of sputum and gastric contents from people ill with a disease characterized by manifestations of tuberculosis. However, upon further laboratory examination of these bacilli, they are proven not to be typical human or bovine types of mycobacteria which are considered the true causative agents of tuberculosis. The atypical forms give unexpected findings in drug susceptibility tests and are usually more resistant to the drugs commonly used in the chemotherapy of tuberculosis. Furthermore, sometimes a few acid-fast colonies appear even after the sputum has been negative for tubercle

bacilli by culture during or after drug therapy.[2] The most important test by which these bacteria are proven atypical is by injection of suitable numbers of the bacilli into guinea pigs. While the typical human and bovine strains produce a progressive disease in the guinea pig, the atypical cultures produce a self limiting infection usually confined to the site of inoculation.[3]

Max Pinner[4] made an important contribution in reporting on the presence of some chromogenic atypical forms which failed to produce progressive disease in guinea pigs. When injected subcutaneously, these bacteria tended to produce small nodules in the subcutaneous tissue. Frequently there were small perforations into the peritoneal cavity, while only rarely did these perforations occur upon administration of Virulent Tubercle bacilli into guinea pigs.

Upon further studies it was shown that a great many of these atypical organisms were chromogenic, and in 1952 Tarshis and Frisch did cultural, pathological and hypersensitivity studies on bacilli obtained from human sources. Their studies showed that the chromogens differ in media required for growth, but this procedure was too complicated for practical use as a diagnostic procedure. These workers found differences in the pulmonary lesion caused in guinea pigs by chromogenic acid-fast bacilli and typical mycobacterium. The lesions caused by the chromogens tend to involve serosa rather than parenchyma, typical tubercles are absent and, in advancing lesions, there is a central necrosis which undergoes abscess formation enclosed by granulation tissue with marked surrounding hyperemia such as is not seen in typical lesions. Hypersensitivity studies further illustrated the difficulty of identifying the chromogens, since there was some cross sensitization due to the sharing of certain antigenic properties in common with human and bovine bacilli.[5]

As previously mentioned, investigators have recently begun to pay more attention to the atypical bacilli, due to their greater frequency of appearance. Some physicians are fearful that the more common usage of anti-tuberculous drugs during the past few years may be partly responsible for the development of these atypical strains. However, one fact which contradicts this belief is the presence of atypical forms in people who have never received any anti-tuberculosis drugs. Also, as reported by Crow and co-workers, many of the atypical cultures are isolated from people who have never been in contact with others given anti-tuberculous chemotherapy.[6] Therefore, it appears more likely that we are becoming more aware of the great number of atypical strains merely because of the increased use of culture methods for diagnostic purposes and drug susceptibility tests.

Besides the work that has been done on comparing pathogenicity in guinea pigs by the different acid-fast bacilli, Timpe and Runyan have compared the virulence of atypical bacilli in other laboratory animals. Employing some of the "atypicals" obtained from diseased human tissue, they studied virulence in mice. They showed that atypical cultures generally associated with human disease were more virulent for mice, in contrast to those which were not as constantly associated with human disease.[7] Pollak and Buhler went even further with studies on animal pathogenicity, proving that atypical acid-fast bacilli which caused human disease failed to produce progressive diseases in guinea pigs, chickens, or rabbits even using massive inocula, while they did produce variable amounts of disease in mice, rats, and Syrian Golden Hamsters.[8]

Other comparisons between human tubercle bacilli and atypical bacilli include the following: They are grossly similar in rate of growth, but

colonies of atypical acid-fast bacilli are commonly convex, smooth and shiny with a soft tenacious consistency and lemon yellow to orange color.[5] This is in contrast to the morphology of the typical forms of mycobacterium. These colonies are commonly rough, adherent to the media and cream or buff-colored.[9] These differences, however, are neither outstanding nor consistent enough to be used in differentiating the various acid-fast bacilli. In addition, the atypical strains can be divided into groups according to their chromogenic properties. Photochromogens develop color only when grown in light, but even the slightest contact with light will cause the development of color. Scoto-chromogens develop an outstanding color, usually shades of orange, no matter whether grown in light or dark. The final subdivision is the Battey type, or non-photochromogens which develop no outstanding color, but remain a light yellow or buff color, much the same as the typical strain, except on long exposure to light at room temperature.

In the medical literature one can find many reports of atypical strains being found in tuberculous lesions of the lungs and other organs.[10,11,12] Since these lesions are often histologically similar to tuberculosis and cause the same clinical symptoms as tuberculosis, one may be led to inquire as to the significance of identifying these atypical forms. The significance of such identification lies in the fact that the prognosis and therapy vary greatly, being dependent upon the identification of the causative agent. The prognosis is usually much more promising when the lesion has been caused by the atypical strains.[3] However, these strains are much more resistant to the chemotherapeutic agents now in use, and Dr. Crow has shown in his studies with seventy patients that there is a relatively low sputum conversion rate (disappearance of

acid-fast bacilli from sputum) among patients with the atypical bacilli. Furthermore, the clinical and roentgenographic response to this therapy was notably unsuccessful. Therefore, if the lesion is established as being caused by an atypical strain, it is best to perform surgery as quickly as possible, in order to remove the lesion while it is still small and fairly well localized.

Finally, it is of great epidemiological importance to make a definite identification of the bacilli present. Data accumulated from family history, histories of marital partners, tuberculin skin tests and x-rays reveal that in infections caused by atypical strains, the danger of transmission of infection in family contacts is reduced to a great extent. The epidemiological problem might then be considered in the light of the following combination of factors: decreased transmissibility, increased resistance to chemotherapy, and the possibility of the failure of surgery to correct the situation. In view of the difference in treatment, the patient is often faced with the possibility of a prolonged stay in the sanatarium, and may leave either without permission or with a "maximal hospital benefit discharge."

The above underlined importance of determining the causative agent of the tuberculous lesion has led to many attempts to develop an accurate and practicable test for the differentiation of the human mycobacterium from other acid-fast strains. In the mid-1940's, Pope and Smith studied a method for differentiation of human and bovine strains based upon the amount of B complex vitamins synthesized by these various bacilli when grown on a vitamin B free media. In their work, they used cultures grown for six weeks at 37° C. on Proskauer and Beck's synthetic media. By means of a microbiological assay procedure, described by Snell and Wright[13],

Pope and Smith checked for the amount of the various B complex vitamins produced by the bacteria under discussion. The results of these assays showed that the human type bacilli synthesized the B vitamins to a greater extent than did the bovine strain tested. The most striking variations were shown by niacin ($B_2$) with the human strain producing fifty times the amount produced by the Bovine strain. Their human strains also produced eleven times as much inositol, seven times as much folic acid and ten times as much para-aminobenzoic acid.[14] A short time later, Bird reported on his findings, which corroborated Pope and Smith's work, but he expressed the belief that only the difference in niacin synthesis was large enough quantitatively to be useful as a laboratory tool in the differentiation of the various mycobacterium strains from the human bacilli.[15]

Though this varibility in the production of niacin gave promise of a method for establishing the various strains of mycobacterium, the microbiological assay method as utilized by Pope, Smith and Bird was far too complicated and time consuming to be of practical nature as an aid to the clinician. It was only in November of 1956 that Kiyoshi Konno reported the use of a simple chemical method for establishing the amount of niacin produced by various mycobacterium. This method was the one developed originally by Feinstein for estimating the niacin content of flour.[16] This test was based on the Konig reaction[17], in which pyridine compounds react with cyanogen bromide and primary or secondary amines to produce complex colored compounds. In his researches, Konno used laboratory strains of mycobacteria, tubercle bacilli from patients and atypical acid-fast bacilli which he had grown on Lowenstein-Jensen solid culture media for one to three months. Colonies taken from solid medias were added to one milliliter of 4% aniline in 95% ethyl alcohol and one milli-

liter of 10% cyanogen bromide solution. If the colonies contained niacin, the solution turned a canary yellow color. Then in order to prove that the color was developing due to the niacin and was not coming from the color of the bacteria, he left out either the aniline solution or cyanogen bromide solution; under these circumstances no color appeared. By this qualitative method he, too, concluded that only human type bacilli produced niacin in considerable quantities.[18]

In order to take advantage of this observation in the development of a practical laboratory test, several factors still required attention, namely, a determination of the amount of culture material required to give a clear test and secondly an attempt to quantitate the color development reaction by means of a photoelectric colorimeter.

## II

## EXPERIMENTAL

In view of the qualitative nature of the work, reported by Konno, it was decided to attempt to quantitate the differences in niacin synthesis in various mycobacteria by measuring optical densities of the yellow solutions formed upon the addition of aniline and cyanogen bromide solutions to the bacilli. First, it was necessary to set up a standard solution containing a known amount of niacin (10 mg. per 500 ml. distilled water). From this, dilutions were prepared to give the desired concentrations. The aniline and cyanogen bromide were then added to these known solutions. Two blank solutions were made in order to determine how much the optical density would vary when the solutions were read against the different blanks. It was also necessary to establish whether the type of blank used was a critical factor in the photometric readings. This proved not to be

the case. Blank I contained 1 ml. of niacin solution, 1 ml. of distilled water, and 1 ml. of aniline, while Blank II contained 1 ml. of distilled water, 1 ml. of aniline and 1 ml. of cyanogen bromide. Then, with two niacin solutions (8$\gamma$ per ml. and 16$\gamma$ per ml.) optical density was determined at different wave lengths from 400$\lambda$ to 600$\lambda$, in order to discover at which wave length the difference in optical densities would be greatest, and which wave length would therefore be optimal for all future determinations. This wave length was found to be 445$\lambda$. (Table I) Also, readings were taken immediately after adding the solutions and one hour after their addition in order to establish whether color development varied with time. It was discovered that time played a negligible roll in the development of color. Finally, curves were set up plotting optical density against the known niacin solutions. (Graph I and II.)

After this, a preliminary study was done with human tubercle bacilli grown on a malic acid medium for six to eight weeks. The bottle containing medium and culture was first autoclaved at fifteen pounds pressure for twenty minutes; the mixture was then filtered and the filtrate saved. The autoclaved bacilli were then washed and dried to constant weight and various quantities (25-40 mg.) were tested for niacin content. Though this strain was known to produce considerable amounts of niacin, the optical density determinations of the niacin content of the dead bacilli showed them to be virtually devoid of niacin. (Graph III) It was assumed, therefore, that the niacin must have been transfered from the bacteria to the medium (or filtrate) during autoclaving. Accordingly, we next tested the filtrate, which had in the meantime been sterilized by filtration and kept refrigerated in order to keep out contaminants which might have altered the niacin content. The different dilutions of the filtrate

gave fairly constant amounts of niacin per ml. (Graph IV--also Table II) Unfortunately, in order to make this truly quantitative for niacin concentration per mg. of bacteria, it would have been essential to know the total weight of the dried bacilli and the volume of the media. Since we were searching for the rapid and simple test as well as for an accurate one, it was decided to try another procedure which had greater possibilities of satisfying all three requirements.

The method that was finally adopted was one which utilized cultures grown for three to six weeks at 37° C. on Trudeau Society Medium. After the colonies were fairly abundant, about 20-40 mg. were carefully scraped off the medium into dried, pre-weighed bottles. These bottles were then autoclaved under fifteen pounds pressure for twenty minutes. In removing the colonies from the tube, care was taken not to take along any of the medium as this would have given an incorrect weight for the dried colonies. The bottles containing the bacilli were then dried in the incubator at 37° C. until they reached constant weight, within several tenths of a milligram. One milliliter each of aniline solution and cyanogen bromide was added and the optical density was read against Blank II. From Graphs V, VI and VII, the amount of niacin synthesized by the acid-fast organisms in each weighing bottle was read, and the amount of niacin produced by each milligram of dried bacilli could be calculated. This method was decided upon because it was felt that by transfering the bacteria before autoclaving, we could avoid the problem of whether or not niacin was being released from the bacilli into the media by autoclaving. It was felt that the second method eliminated the major source of error in the procedure. (Table III)

## TABLE I - OPTIMAL OPTICAL DENSITY

| WAVE LENGTH ($\lambda$) | SOLUTION | OPTICAL DENSITY |
|---|---|---|
| 400 | 8 $\gamma$/cc (1) | .21 |
| | 16 $\gamma$/cc (2) | .4 |
| 410 | 1 | .26 |
| | 2 | .52 |
| 420 | 1 | .305 |
| | 2 | .62 |
| 430 | 1 | .33 |
| | 2 | .71 |
| 435 | 1 | .34 |
| | 2 | .74 |
| 440 | 1 | .34 |
| | 2 | .76 |
| 445 | 1 | .34 |
| | 2 | .77 |
| 450 | 1 | .31 |
| | 2 | .77 |
| 500 | 1 | .07[illegible] |
| | 2 | .218 |
| 600 | 1 | .005 |
| | 2 | .01 |

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## TABLE II

a) Niacin in dried colonies from malic acid media

| Sample # | Weight (mg.) | Optical Density | $\gamma$ Niacin per Sample | $\gamma$ Niacin/mg. of Sample |
|---|---|---|---|---|
| 1 | 25 | .029 | .45 | .018 |
| 2 | 30 | .048 | .76 | .025 |
| 3 | 35 | .051 | .8 | .023 |
| 4 | 40 | .051 | .65 | .016 |
| 5 | 45 | .046 | .71 | .015 |

b) Niacin/ml. of Malic Acid Media

| Cuvette # | ML Media | ML.$H_2O$ | Optical Density | $\gamma$ Niacin/ML Media |
|---|---|---|---|---|
| 1 | .1 | .0 | .625 | 12.1 |
| 2 | .9 | .1 | .610 | 13.1 |
| 3 | .8 | .2 | .58 | 13.5 |
| 4 | .7 | .3 | .54 | 14.0 |
| 5 | .6 | .4 | .37 | 11.0 |

TABLE III - NIACIN PRODUCED PER MG. OF DRIED BACILLI

| Strain | Weight Mgm. | Optical Density | Niacin γ/ Total Wt. | γ Niacin/mg. Dried Bacilli |
|---|---|---|---|---|
| 265 | 49.3 | .334 | 5.0 | .12 |
| 265 | 42.7 | .231 | 4.1 | .096 |
| 10115 | 14.8 | .095 | 1.5 | .10 |
| 10115 | 63.2 | .393 | 7.0 | .11 |
| H37Ra | 83.2 | .80 | 17.0 | .20 |
| H37Ra | 12.6 | .125 | 2.1 | .17 |
| $R_1$ | 23.5 | .030 | 0.5 | .021 |
| $R_2$ | 66.3 | .100 | 2.8 | .042 |
| $R_2$ | 42.5 | .15 | 1.7 | .040 |
| $R_3$ | 52.4 | .125 | 2.3 | .045 |
| $R_3$ | 62.2 | .205 | 3.8 | .061 |
| $R_4$ | 29.3 | .00 | 0.0 | .000 |
| $R_5$ | 50.2 | .13 | 2.2 | .044 |
| $R_5$ | 72.5 | .158 | 2.8 | .039 |
| $R_6$ | 95.1 | .20 | 3.7 | .039 |
| $R_6$ | 130.4 | .32 | 6.1 | .047 |
| $R_7$ | 33.1 | .058 | 0.9 | .027 |
| $R_7$ | 20.3 | .025 | 0.4 | .017 |
| $R_8$ | 18.7 | .04 | 0.6 | .032 |
| $R_8$ | 45.3 | .075 | 1.2 | .026 |

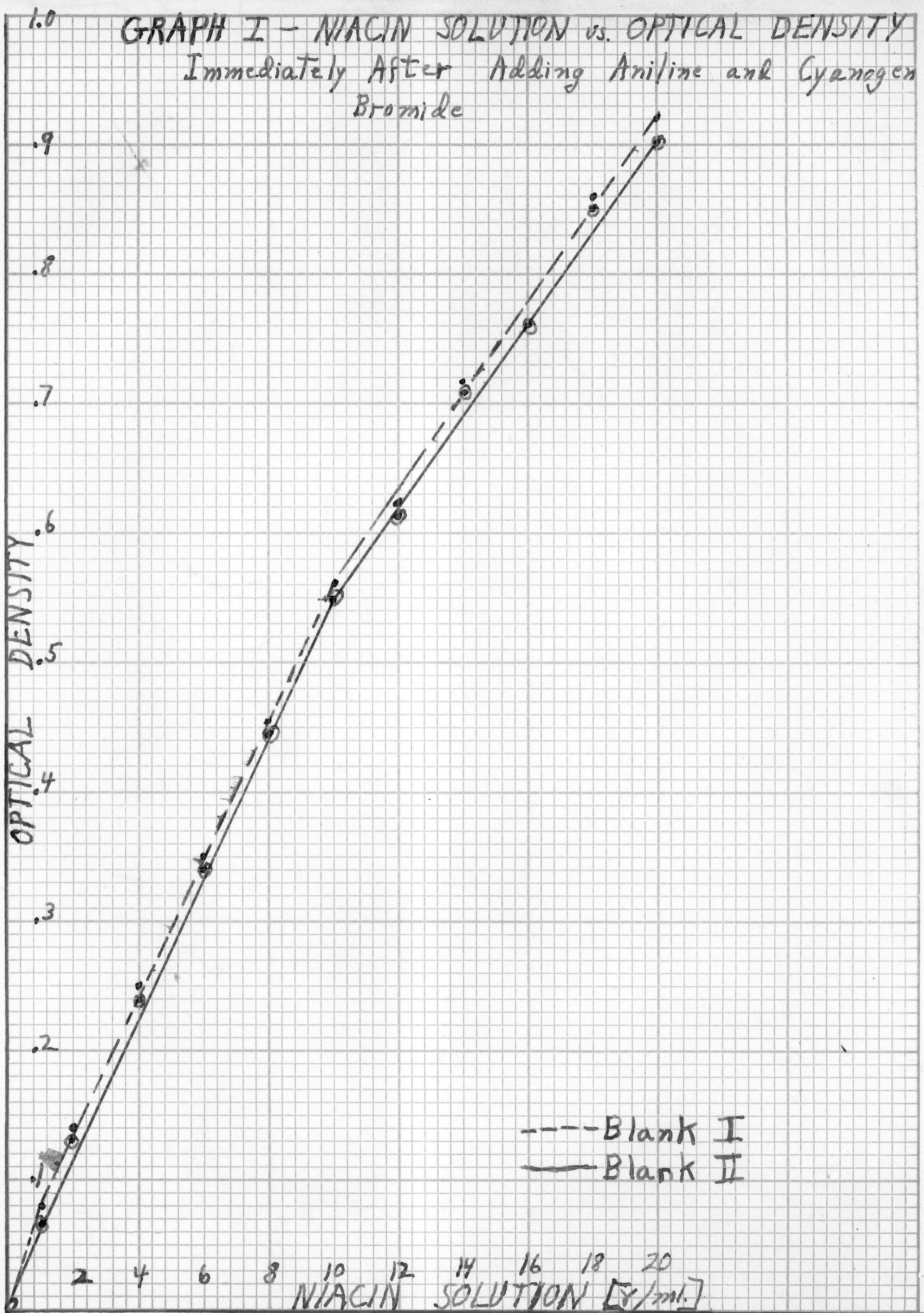
Parenthetically, it is to be noted that somewhat before this was being done here, Konno, Kurzman and Bird used a modified Muller and Fox method,[19] using culture filtrate which had been hydrolized by adding NaOH and heating in a water bath. We were not aware of this until very recently as they reported on this in the April, 1957, issue of American Review of Tuberculosis. With their method, the coenzyme, nicotinamide and nicotinic acid precursor in the filtrate are hydrolized to niacin, and then buffered ammonia solution and cyanogen bromide were added. As described before, a yellow color appeared. The color intensity was then used to find out the amount of niacin which was present. They proved unequivocably that human type bacilli, no matter what the virulance or resistance to drugs, produce more niacin than the other bacilli. Their results showed that the human type produced 13.5 $\gamma$ per ml. of culture filtrate or 6.3 $\gamma$ per mg. of dry bacillary weight while the other mycobacteria did not produce an amount exceeding 2.0 $\gamma$ per ml. or 1.0 $\gamma$ per mg. of dry bacillary weight. Therefore, testing of the filtrate is an adequate quantitative method for differentiation of various strains of mycobacterium.[20]

For our experiment, eleven different strains of mycobacterium were used, eight ($R_1$ - $R_8$) of which were atypical strains of the photochromogen, scoto-chromogen and Battey type; the remaining three strains were the typical human variety of mycobacterium. This method further substantiated the fact that there is as marked difference in the amount of niacin synthesized by the human and atypical varieties of acid-fast bacilli. The least amount of niacin produced by any of the human strains was .096 $\gamma$ per mg. of dried bacillary weight, but the most common amount produced was in the range of .11 - .13 $\gamma$ per mg. of dry bacilli. The atypical forms tested produced much smaller amounts of niacin, somewhere within the range of .02 - .04 $\gamma$ of niacin per mg. of bacilli.

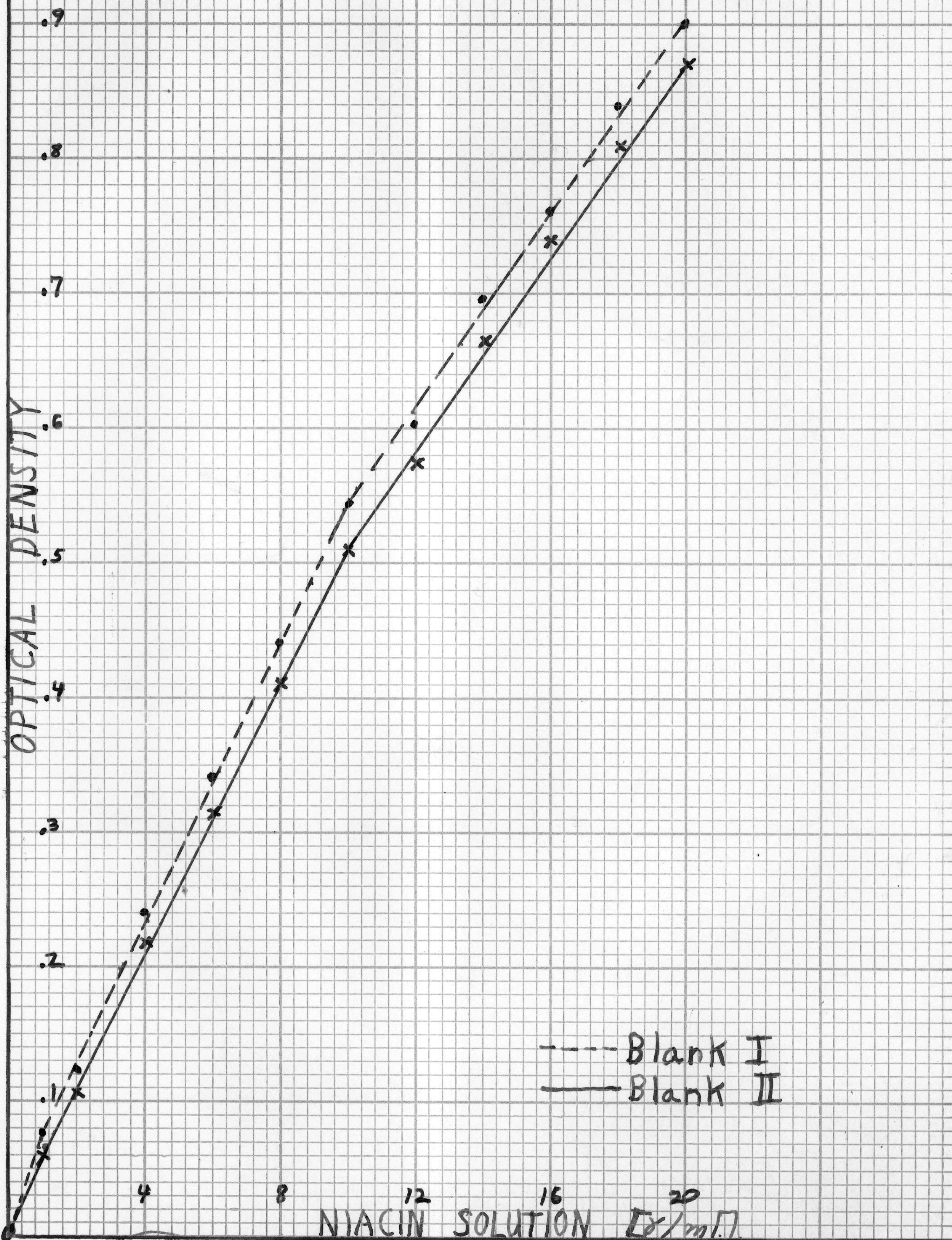
As indicated above, the difference in niacin content between typical human and other strains of mycobacteria appears to be significant and capable of laboratory determination. Though the test requires further clarification and development, this difference in niacin content and the above described method for establishment of the niacin content appear to hold promise as a method for clinical laboratory tests for the differentiation of the mycobacteria. Such a test would offer aid to the clinicians in establishing the etiology of a granulating lesion produced by acid-fast bacilli.

The method above described, with further development, would appear to satisfy the basic requirements set forth previously in this paper: 1) Simplicity in that no elaborate equipment or complicated procedures are involved; 2) Rapid, in that an adequate amount of material can be obtained after three to four weeks of colony growth; 3) Quantitative, in that one, with simple calculations, can establish to within tenths and one hundreths of a microgram the niacin produced. This study indicates a reproducibility of results which would allow its use as a laboratory procedure.

This test appears to hold promise, therefore, as a practical laboratory procedure for the differentiation of mycobacteria, a differentiation which would offer aid to the clinician.


GRAPH I - NIACIN SOLUTION vs. OPTICAL DENSITY
Immediately After Adding Aniline and Cyanogen Bromide
OPTICAL DENSITY
NIACIN SOLUTION [γ/ml.]
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
2
4
6
8
10
12
14
16
18
20
----Blank I
——Blank II

Graph II – Niacin Solutions Vs. Optical Density
One Hour after adding Aniline and Cyanogen Bromide



FORM C3

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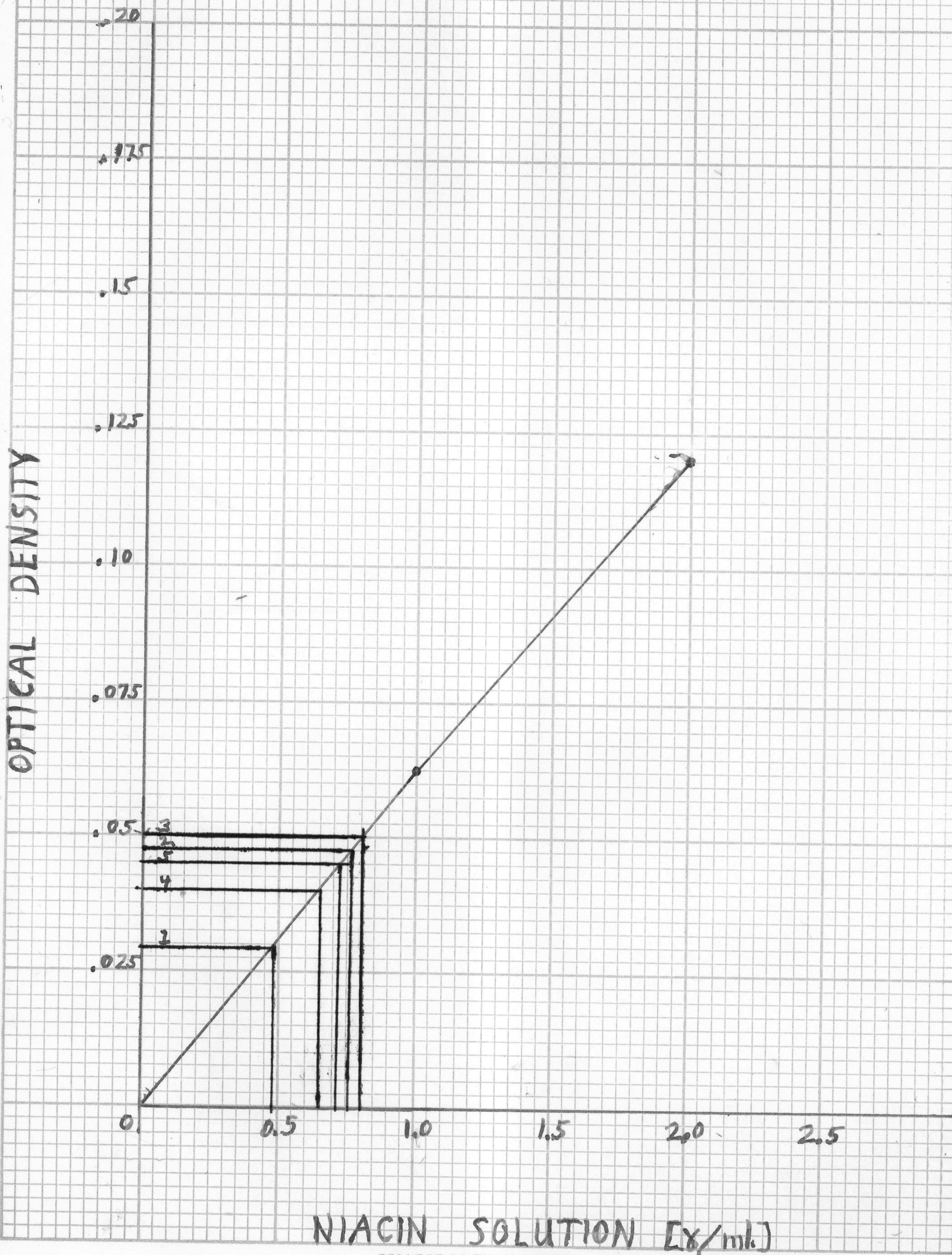


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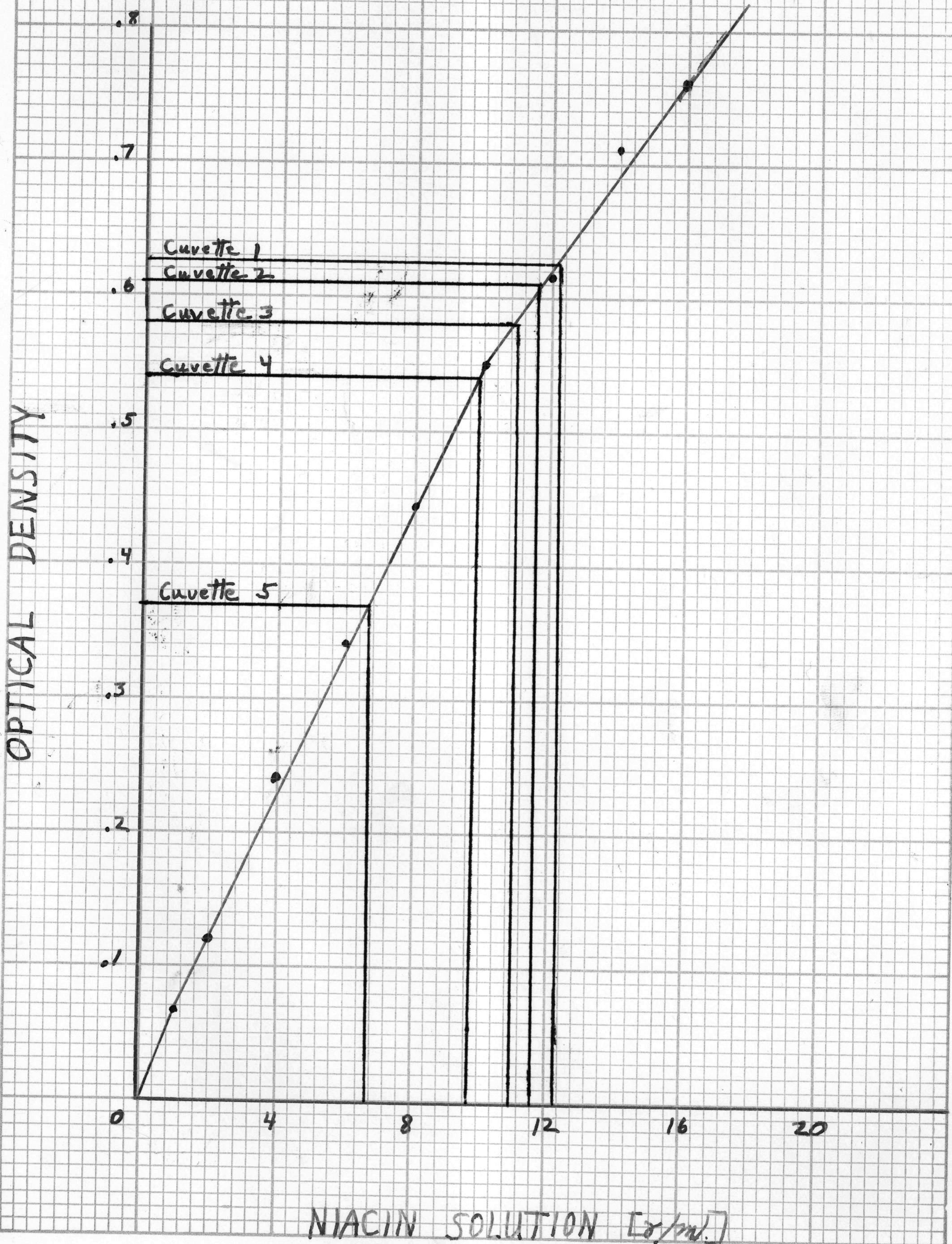
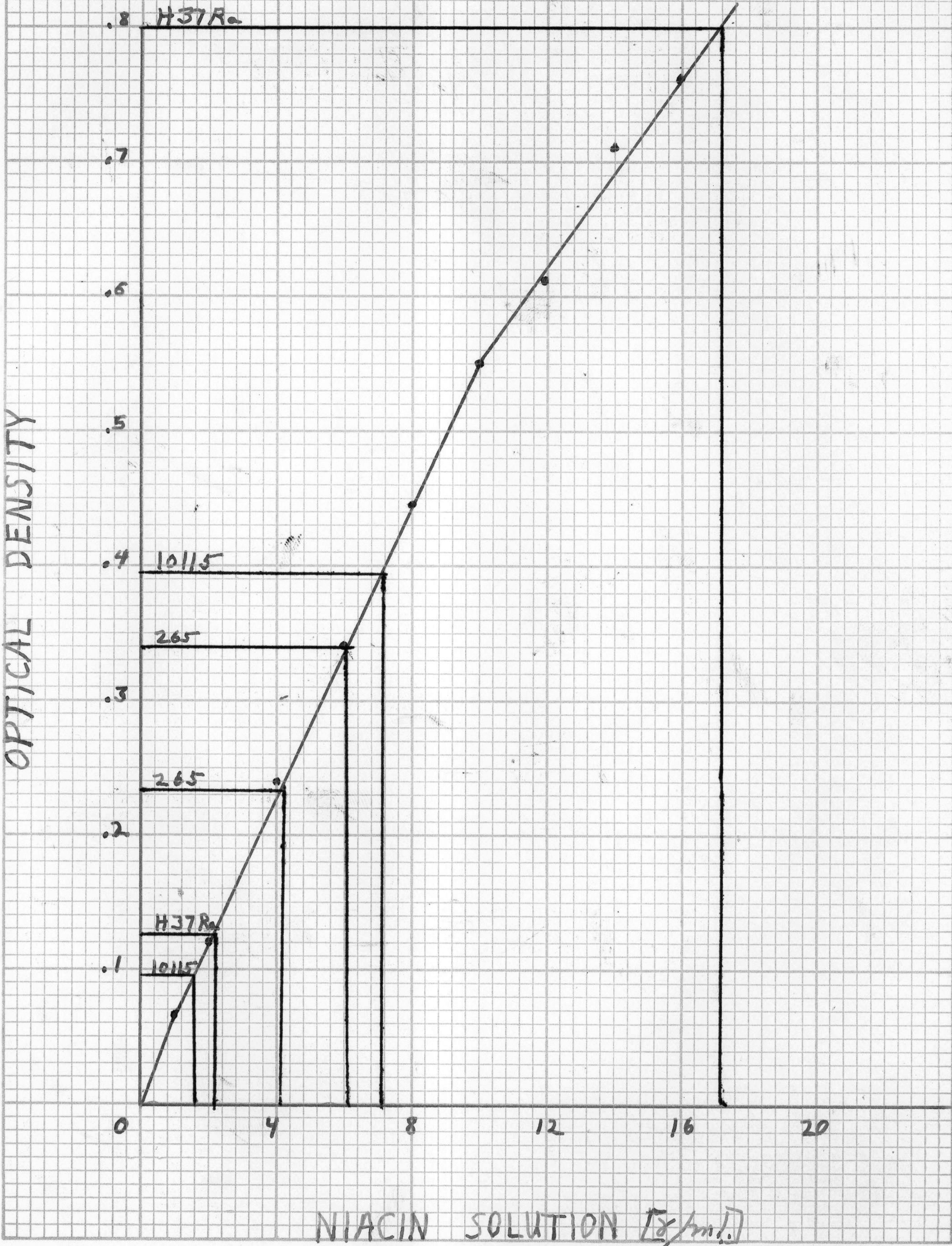
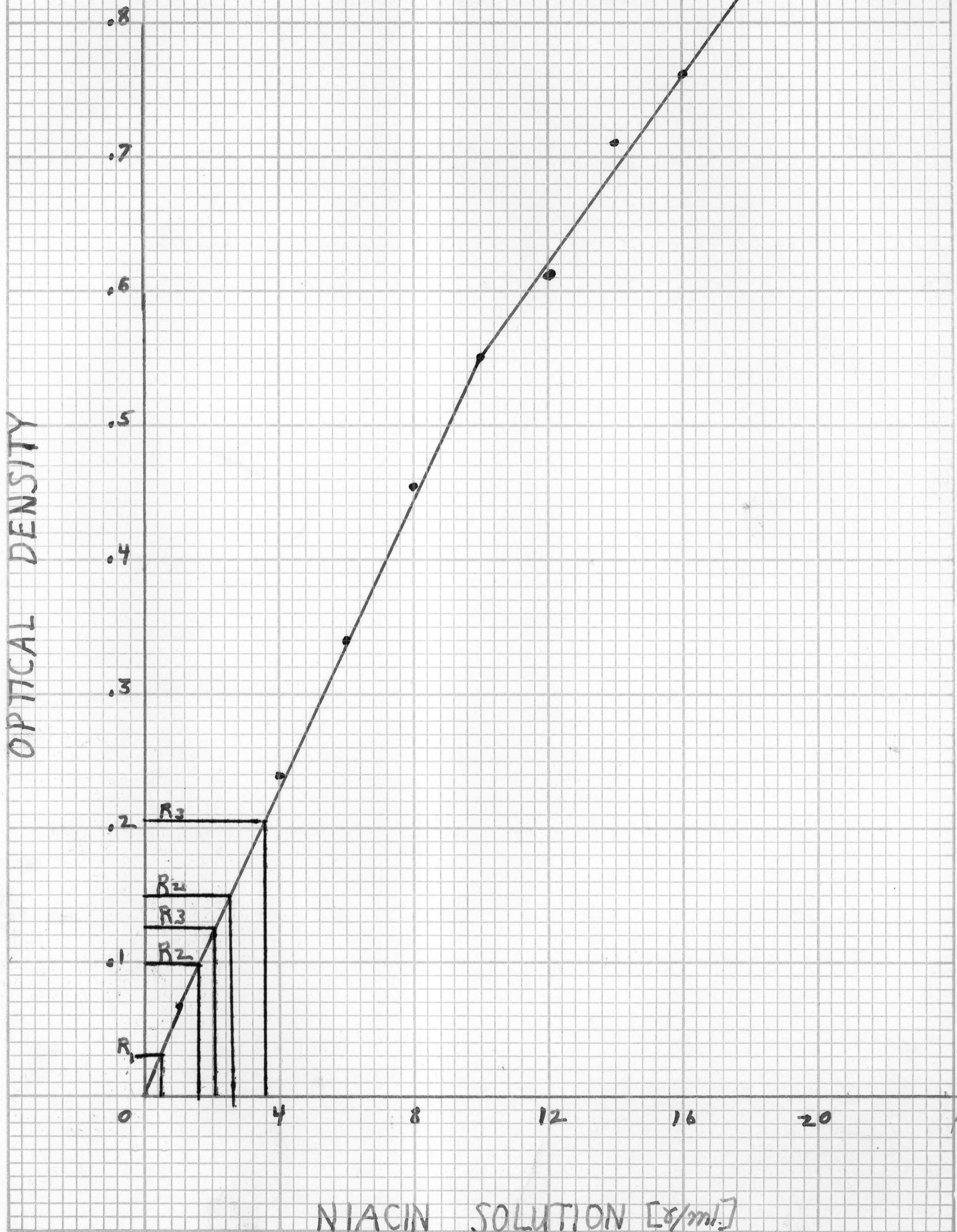

Graph IV - Niacin Per Milliliter of Malic Acid Media [Table II-b]
OPTICAL DENSITY
.8
.7
.6
.5
.4
.3
.2
.1
0
4
8
12
16
20
Cuvette 1
Cuvette 2
Cuvette 3
Cuvette 4
Cuvette 5
NIACIN SOLUTION [γ/ml.]

6




Graph VI Quantity of Niacin Synthesized by Atypical Forms of Mycobacteria [Table III]
OPTICAL DENSITY
.8
.7
.6
.5
.4
.3
.2
.1
R3
R2
R3
R2
R1
0
4
8
12
16
20
NIACIN SOLUTION [γ/ml.]

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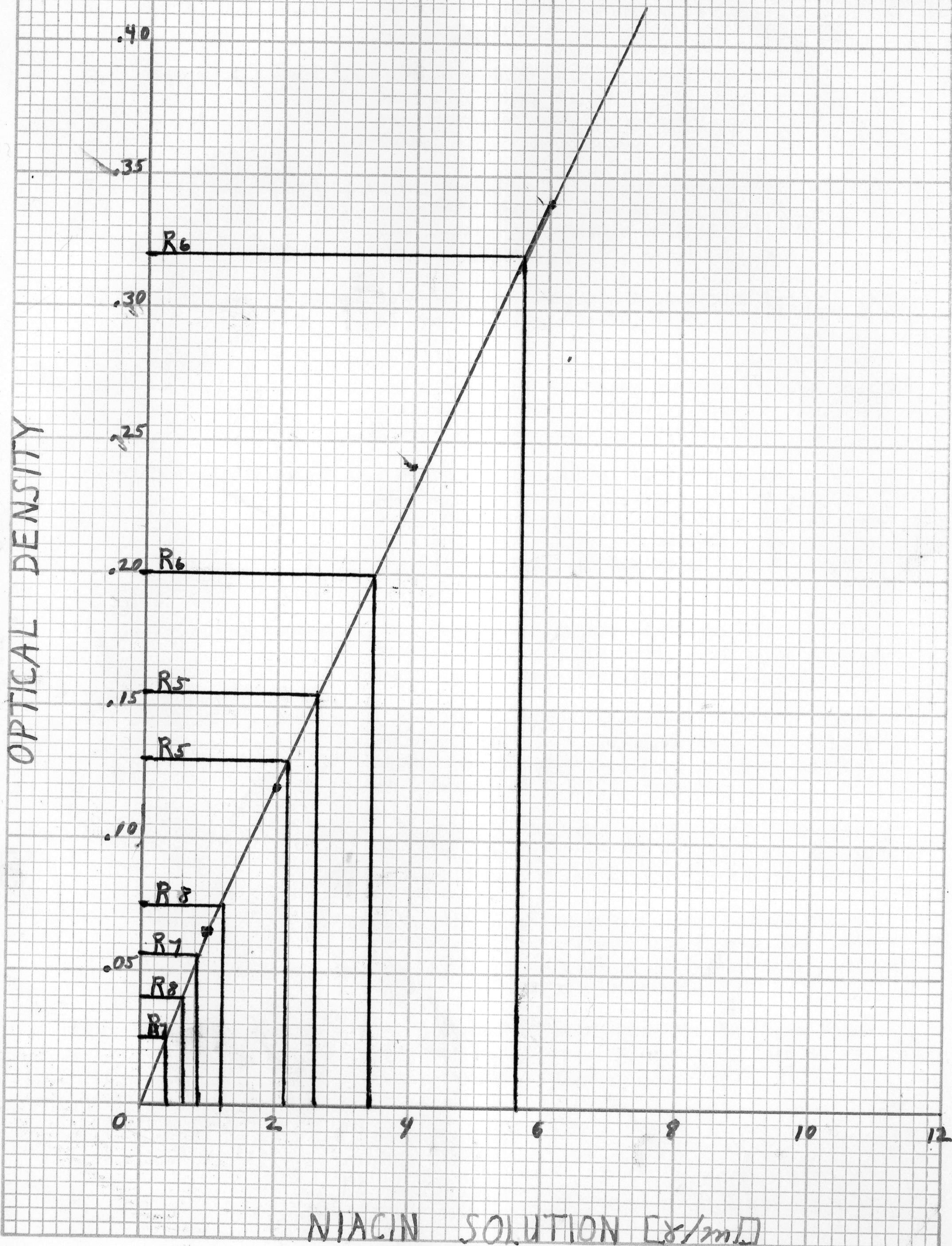
Graph VII Quantity of Niacin Synthesized by Atypical forms of Mycobacteria [Table III]

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