A STUDY OF AN ENZYMATIC METHOD FOR THE DETERMINATION OF NICOTINIC ACID IN FOODS

A Thesis

by

Charles Adam Nichol

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GENERAL INTRODUCTION

The identification of nicotinic acid as the pellagra-preventative factor has necessitated the development of accurate methods for its estimation. Most chemical methods are colorimetric procedures based upon the Konig reaction (52) in which the pyridine ring is ruptured with cyanogen bromide. The slight color produced is intensified by the addition of an aromatic amine. This reaction is not specific for nicotinic acid but is given by any alpha-unsubstituted pyridine compound (34). The natural-occuring nicotinamide and related compounds which are biologically active must be converted to the free acid by hydrolysis with acid or alkali prior to the application of the colorimetric procedure. As a result of this hydrolysis many materials give highly colored extracts containing interfering substances.

The numerous procedures for the chemical determination of nicotinic acid are chiefly concerned with the removal of pigments and compensating for the non-specificity of the Konig reaction. Consequently, the procedures are limited in their application since they are designed to overcome the specific interfering substances present in the particular sample being

analysed. For example, methods for the analysis of nicotinic acid in animal tissues do not give satisfactory results when applied to cereals. By standardizing the technique, an individual worker can obtain comparable results but the report of the 1944-45 committee on nicotinic acid assay (79) indicates a lack of agreement in the results obtained by different laboratories analysing the same samples.

Microbiological assays possess the advantage over chemical methods in that turbidity and color do not interfere. Although considered to be more specific than chemical methods, microbiological procedures are subject to error due to variations in technique and to the presence of non-nicotinic acid growth-promoting substances in cereals (15, 62, 2, 14). The time required to carry out the microbiological methods makes a more rapid method desirable.

Much effort has been directed to eliminating the sources of interference in the chemical procedures but comparatively little has been done to improve the specificity of the reaction, and so give the procedure wider application in the analyses of both plant and animal products. An adaptive enzyme capable of specifically destroying nicotinic acid can be obtained from bacteria which will grow on a simple synthetic

medium containing nicotinic acid as the sole source of carbon and nitrogen. Allinson (1) utilized this biological specificity of bacterial enzymes in a method for the determination of nicotinic acid in blood. This makes possible the measurement of the non-nicotinic acid chromogenic materials which cannot be accomplished by chemical procedures. After correcting for the interfering color the method gives a reliable estimate of the nicotinic acid content of a sample.

This study is concerned with the preparation and characterization of such bacterial enzymes and their application to the chemical determination of nicotinic acid in foods.

PART I

STUDIES ON THE ENZYMATIC DESTRUCTION OF NICOTINIC ACID

PART I HISTORICAL INTRODUCTION

Karstrom (43) was the first to differentiate between adaptive and constituted enzymes in bacteria. Dubos and Miller (21) isolated from soil a Gram-positive pleomorphic bacterium capable of decomposing creatinine which they designated NC (neutral culture). An adaptive enzyme produced by this organism was used for the analysis of creatine and creatinine in tissues by Miller, Allinson and Baker (68). More recently, Allinson (1943) used the NC organism to produce an enzyme which specifically destroyed nicotinic acid. When grown on a medium containing creatinine as the only source of carbon and nitrogen enzymes capable of attacking nicotinic acid were not produced.

Koser and Baird (53) made an extensive investigation of bacteria which destroy nicotinic acid. They report that Serratia marcescens and Pseudomonas fluorescens can grow on a synthetic medium in which nicotinic acid is the only organic compound.

EXPERIMENTAL

1. Selection of an organism

To find the organisms which are most active in destroying nicotinic acid the properties of several cultures were compared. Colorimetric determination of the nicotinic acid in a medium showed that the growth of the organism can serve as a measure of the nicotinic acid destroyed. The rate of growth and some of the nutrient requirements of the cultures listed in Table I were studied.

Table I - Source and designation of bacterial cultures.

Culture designation	Source	Isolated by
NC (neutral culture)	soil	Dubos and Miller(21)
CO (contaminant)	water	Author
Serratia marcescens	soil	*Gray
Pseudomonas fluorescens 1	water	Gray
2	water	Gray
3	water	Gray
4	water	Gray
12	water	Koser and Baird (53)
30	water	Koser and Baird (53)

* Department of Bacteriology, Macdonald College, P.Q.

An unsterile flask of liquid medium developed

a bacterial growth which was encouraged by incubation. The active organism, CO, which destroyed the nicotinic acid in the medium was isolated but was not classified.

Miller, Allinson and Baker (68) found it difficult to culture NC when the medium was made up with Chicago tap water so they prepared an artificial tap water approximating the composition of that in New York city. Growth of NC was unsatisfactory with Macdonald College tap water and was improved only slightly with artificial tap water. Allinson (1) incubated NC at 37.5°C. The optimum temperature for Pseudomonas fluorescens was 20° to 25° and for Serratia marcescens 25° to 30°C. The organism active at room temperature was preferable since special incubation was not required during the assay procedure.

To find the culture best able to obtain nitrogen from the pyridine ring, three media were compared: (I) Allinson's medium containing 200 mg. Difco yeast extract per liter and made up with artificial tap water; (II) The Koser and Baird medium containing $(NH_4)_2HPO_4$ and nicotinic acid as the sources of nitrogen; (III) The Koser and Baird medium containing Na_2HPO_4 and nicotinic acid as the only source of nitrogen. Inoculation from nutrient agar slopes was made into

50 ml. of medium in 250 ml. Erlenmeyer flasks. After 72 hours aeration on an automatic shaker at a constant temperature of 25°C the growth on each medium was compared, with the results indicated in Table II.

Table II - Comparison of growth on medium I, II, and III after 72 hours.

Culture designation	Medium I	Medium II	Medium III
NC	xx	x	x
CO	xxx	x	-
Serratia marcescens	xxx	x	x
Pseudomonas fluorescens 2	xxxx	xxx	xxx
" 4	xx	x	x
" 12	xxxx	xxxx	xxxx
" 30	xxxx	xxxx	xxxx

- indicates no growth, x indicates very light turbidity, xxxx indicates heavy turbidity and complete destruction of nicotinic acid.

Serratia marcescens and CO required 7 to 8 days to completely destroy all the nicotinic acid in medium I whereas Pseudomonas fluorescens 2 had destroyed the nicotinic acid in medium III in 5 days. Greatest activity was shown by cultures 12 and 30 which destroyed the nicotinic acid in medium III within 36 hours. The mineral requirements of Pseudomonas

<u>fluorescens</u> 12 and 30 were easily met since each was found to grow well on media made up with tap water or distilled water plus $MgSO_4$. These two cultures were selected for further study since they destroy nicotinic acid very rapidly at room temperature.

2. Culture of the organism

Growth was satisfactory on a medium made up of

Na_2HPO_4	2.0 gm.
KH_2PO_4	1.5 "
NaCl	5.0 "
$MgSO_4$	0.1 "
Nicotinic acid	2.0 "
Distilled water	1000 ml.

After neutralizing the nicotinic acid the hydrogen-ion concentration was adjusted to pH 6.8 - 6.9 with NaOH. Stock cultures were maintained on agar slopes of this medium and on nutrient agar. The organisms became adapted to growth on the nicotinic acid medium by repeated transfers in liquid medium. This activity was maintained for long periods in cultures growing on nicotinic acid agar. <u>Ps. fluorescens</u> 12 and 30 were repeatedly transferred on nicotinic acid-agar slopes over a period of six months without

loss of activity.

A characteristic of growth on nicotinic acid agar was the production of a bright green pigment within 18 to 24 hours. The color diffused throughout the agar and darkened to a reddish brown by the end of 48 hours. An attempt to establish a quantitative relationship between the pigment and small amounts of nicotinic acid proved to be unsatisfactory.

3. Effect of carbon dioxide on growth

A different growth which lacked the normal color was observed in one flask which had tipped over on the shaker so that the cotton plug became wet. This growth under conditions of faulty aeration suggested that carbon dioxide was utilized for growth. The source of carbon in the medium was restricted to nicotinic acid.

Four bubbler-tubes containing nicotinic acid medium were innoculated with Ps. fluorescens 50. Two of the tubes received normal air while the other two received air from which the carbon dioxide was removed by washing with 50% potassium hydroxide and soda lime.* As the turbidity of the

*Appreciation is expressed to Dr. A. S. Perlin for the use of his apparatus.

culture increased, the nicotinic acid in the medium was determined by the colorimetric assay. (Table III).

When the nicotinic acid was completely destroyed, the bacteria were thrown down and resuspended in distilled water, one-twentieth of the original volume. Five days later the cells grown in the presence of carbon dioxide could be evenly suspended by shaking but the cells grown in the absence of carbon dioxide had coagulated.

Table III - Effect of carbon dioxide on the rate of growth.

Observation	Rate of Growth With CO_2	Without CO_2
First turbidity(i)	18 hours	37 hours
(ii)	14 "	40 "
Complete destruction(i)	30 "	60 "
of nicotinic acid (ii)	34 "	65 "

The absence of carbon dioxide caused a definite inhibition of growth of Ps. fluorescens in nicotinic acid medium and decreased the viability of the organism. Agitation of the medium to improve aeration facilitates growth.

4. Preparation of the bacterial suspension

Transfers of Ps. fluorescens 30 were made

from nicotinic acid agar slopes to 50 ml. of medium in 250 ml. Erlenmeyer flasks. After 24 hours on the shaker 10 ml. of this culture was inoculated into 500 ml. of the same medium in a 2 l. flask. All of the nicotinic acid was destroyed within 18 to 24 hours when the medium was agitated. When only a weak color test for nicotinic acid was obtained the cells were thrown down, washed twice in fiftieth molar sterile neutral buffer and resuspended in sterile water one-twentieth the volume of the culture medium.

Bacterial suspensions stored at 5°C retained their activity for more than two months. The pH of the stored suspensions was observed to increase gradually with age to approximately pH 8. This change did not appreciably affect the activity of the organism. When stored in fifteenth molar neutral phosphate buffer the bacteria coagulated and could not be evenly dispersed by shaking. The viscosity of the buffered suspension was also observed to increase.

5. Method of testing the activity of a suspension

The activity of a suspension is a measure of its ability to destroy nicotinic acid and is

expressed as the amount of nicotinic acid which a given amount of the suspension will destroy in a given time. Allinson calculated that 1 ml. of the NC suspension could decompose at least 200 micrograms of nicotinic acid in 30 minutes.

To 5 ml. aliquots containing 50 micrograms of a standard nicotinic acid solution was added 0.5 ml. of 0.2 M phosphate buffer pH 6.0 and 0.05 ml. of the bacterial suspension. The cells were dispersed by shaking and the tubes allowed to stand at room temperature for 10, 15, 20, 25 and 30 minutes respectively. The activity of the enzyme was stopped after each period by placing the tube in a water bath at 90°C for five minutes. A heated or inactive suspension was added to a similar aliquot. The cells were removed from each aliquot by centrifuging at 10,000 r.p.m. for 15 minutes using the six place head for the International Multispeed attachment. 5 ml. of the clear supernatant was taken for color development as described in the analytical procedure. Blank corrections were made and the amount of nicotinic acid destroyed was plotted against the time required (Fig.I).

It was found that 50 micrograms of nicotinic acid were destroyed by 0.05 ml. of a suspension of Ps. fluorescens 30 in fifteen minutes at room temperature.

6. Specificity of the active suspension

Using aliquots containing 50 micrograms of nicotinamide a similar test for the activity of the suspension was carried out. The amount of nicotinamide destroyed during one hour was observed. The results are presented in Fig. I.

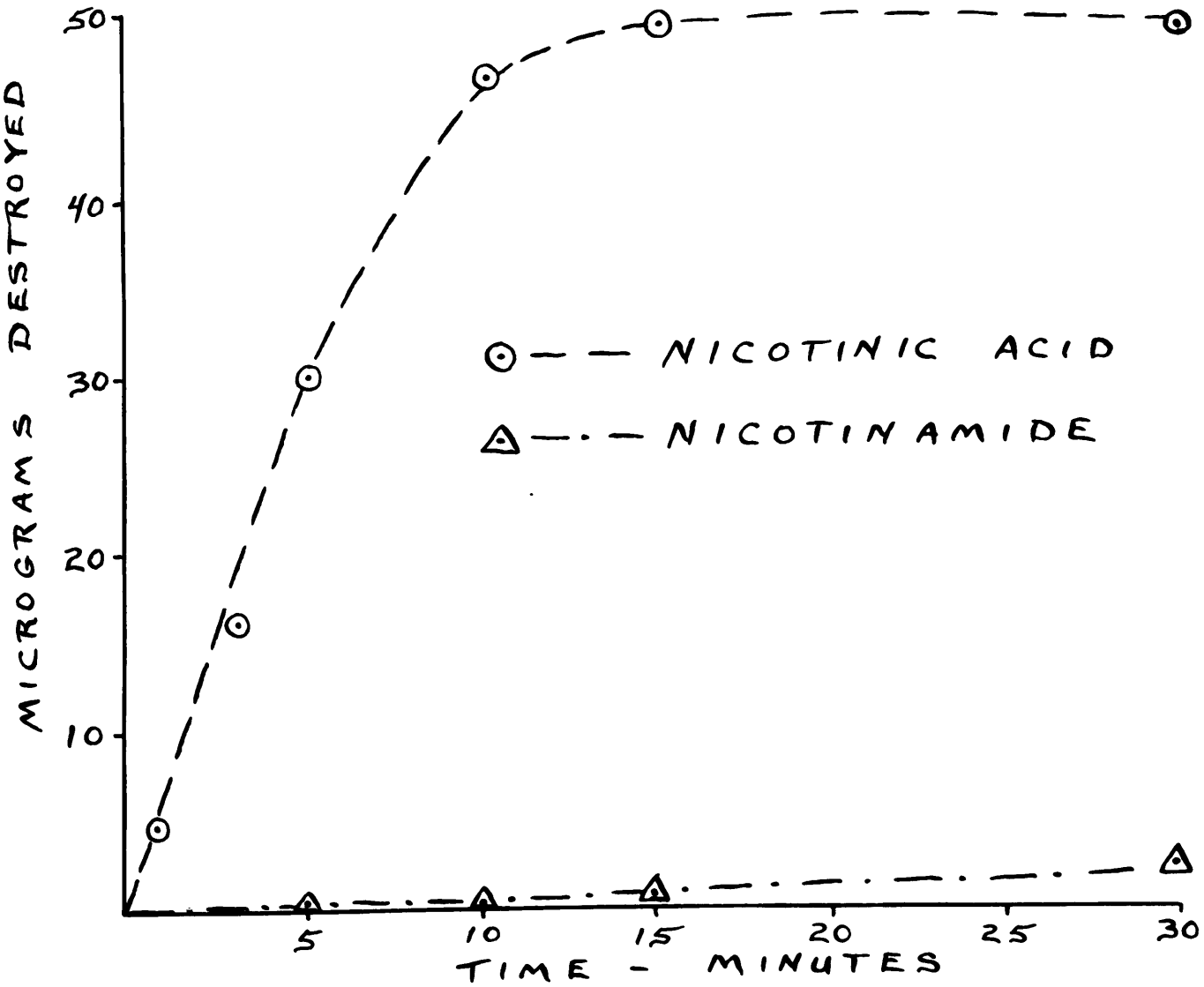

Fig. I - Rate of destruction of nicotinic acid and nicotinamide in solutions containing 50 micrograms of each.

Under the conditions of the assay procedure the enzyme was specific for nicotinic acid. The same amount of the active suspension which destroyed 50 micrograms of nicotinic acid in 15 minutes decomposed only 7 micrograms of nicotinamide at the end of one hour.

7. pH of optimum activity

Aliquots containing 50 micrograms of nicotinic acid were buffered over a range of hydrogen-ion concentrations from pH 3 to 9 and 0.05 ml. of active suspension was added to each tube and allowed to act for 15 minutes. After heating at 90°C for five minutes the cells were thrown down by centrifuging and 5 ml. aliquots of the supernatant were taken for the color reaction. The intensity of the color developed is a measure of the activity of the suspension at each pH. (Fig. II).

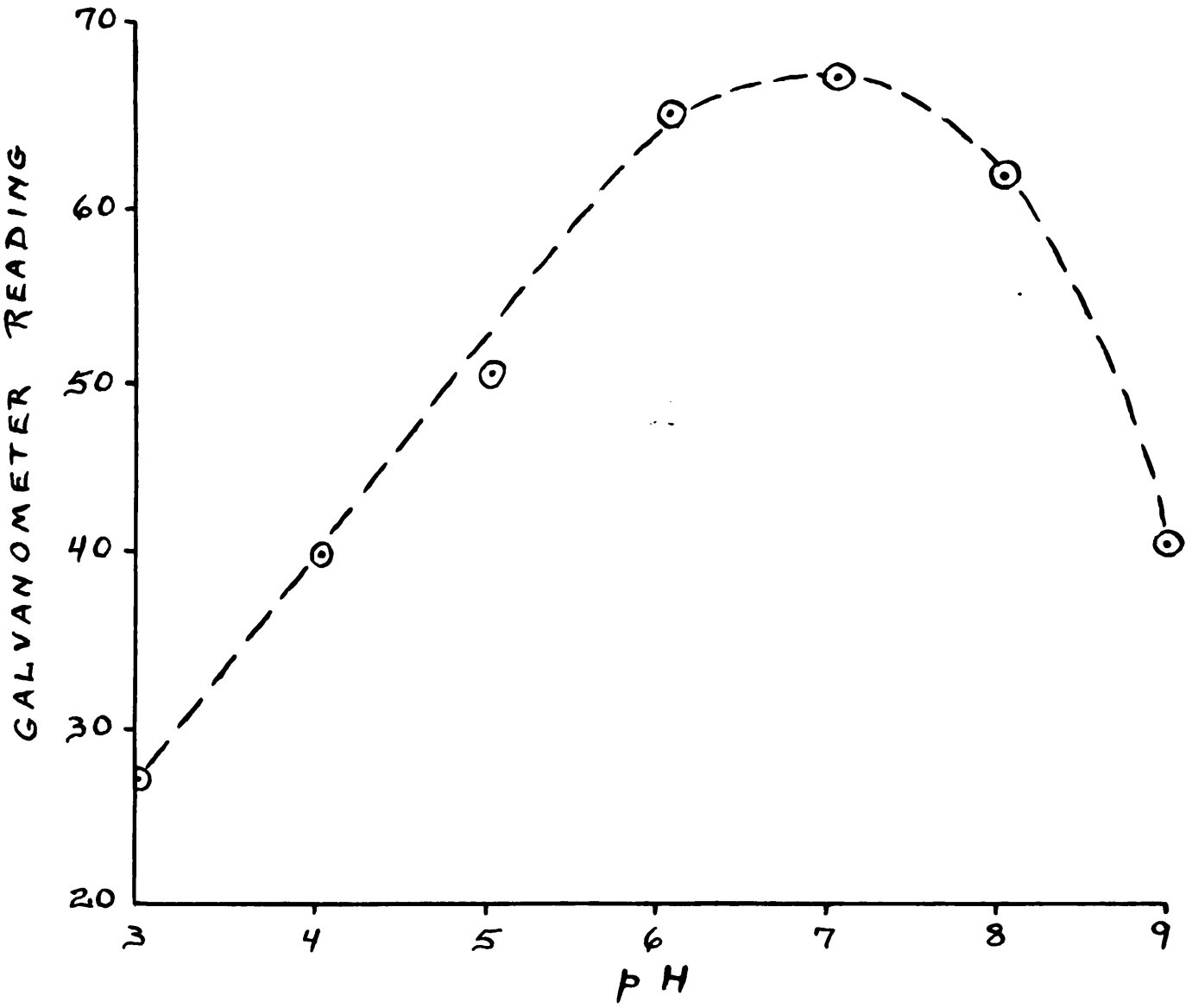

Fig. II - Relationship of the enzyme activity to pH

Optimum activity of the enzyme was at pH 7 but the activity was maintained over a fairly wide range. For the assay procedure pH 6.0 was chosen since color development could then be carried out without further adjustment of the hydrogen-ion concentration.

8. Effect of cell destruction on enzyme activity

Complete removal of the bacterial cells was essential before applying the color reaction and this

required centrifuging at high speed. A cell-free extract of the enzyme could simplify the assay procedure. Dubos and Miller (21) were unable to extract an active soluble enzyme from the PC bacteria.

To study the effect of cell destruction on the activity of the enzyme a suspension of Ps. fluorescens 30 was alternately frozen and thawed, the cycle being repeated eight times within twenty-four hours. This procedure ruptured the bacterial cells. The test for enzyme activity was carried out as described on the intact and the ruptured cells. (Table IV).

Table IV - Effect of cell destruction on enzyme activity

Time	Micrograms nicotinic acid destroyed by (i) Normal cells (ii) Ruptured cells	
5 minutes	29.6	5.4
10 "	47.0	9.9
15 "	50 -	17.0
30 "	-	22.3

Although this experiment indicated that repeated freezing and thawing destroyed 75 - 80 % of the normal activity of the suspension, it did not preclude the possibility that an active extract of the enzyme may yet be prepared by other techniques.

9. Thunberg experiments

Experiments applying the Thunberg technique were carried out with suspensions of Ps. fluorescens 30 using nicotinic acid, alcohol, glucose, sodium lactate, and sodium succinate as substrates. The results indicated that the enzyme attacking nicotinic acid was not a dehydrogenase although the mechanism of attack was probably oxidative. However, the presence of nicotinic acid inhibited the dehydrogenation of alcohol, glucose, lactate and succinate.

10. Warburg experiments

Warburg experiments were carried out at 27°C using 0.5 ml. of M/5 phosphate buffer at pH 7.0 and 1.0 ml. of a suspension of Ps. fluorescens 30. 0.1 ml. of M/10 nicotinic and M/10 sodium azide and hydroxylamine hydrochloride respectively were added from the side arms. The centre well contained 0.2 ml. of 50% KOH for the absorption of carbon dioxide. The contents of each vessel was made up to 3.0 ml. with sterile water and the final concentration of the inhibitors was M/60.

The mode of attack by the enzyme on nicotinic acid was oxidative. The results presented in Fig. III indicate that oxygen uptake by Ps. fluorescens 30 in the

presence of nicotinic acid was inhibited by sodium azide and hydroxylamine hydrochloride which are known to be poisons for heavy metal catalysts.

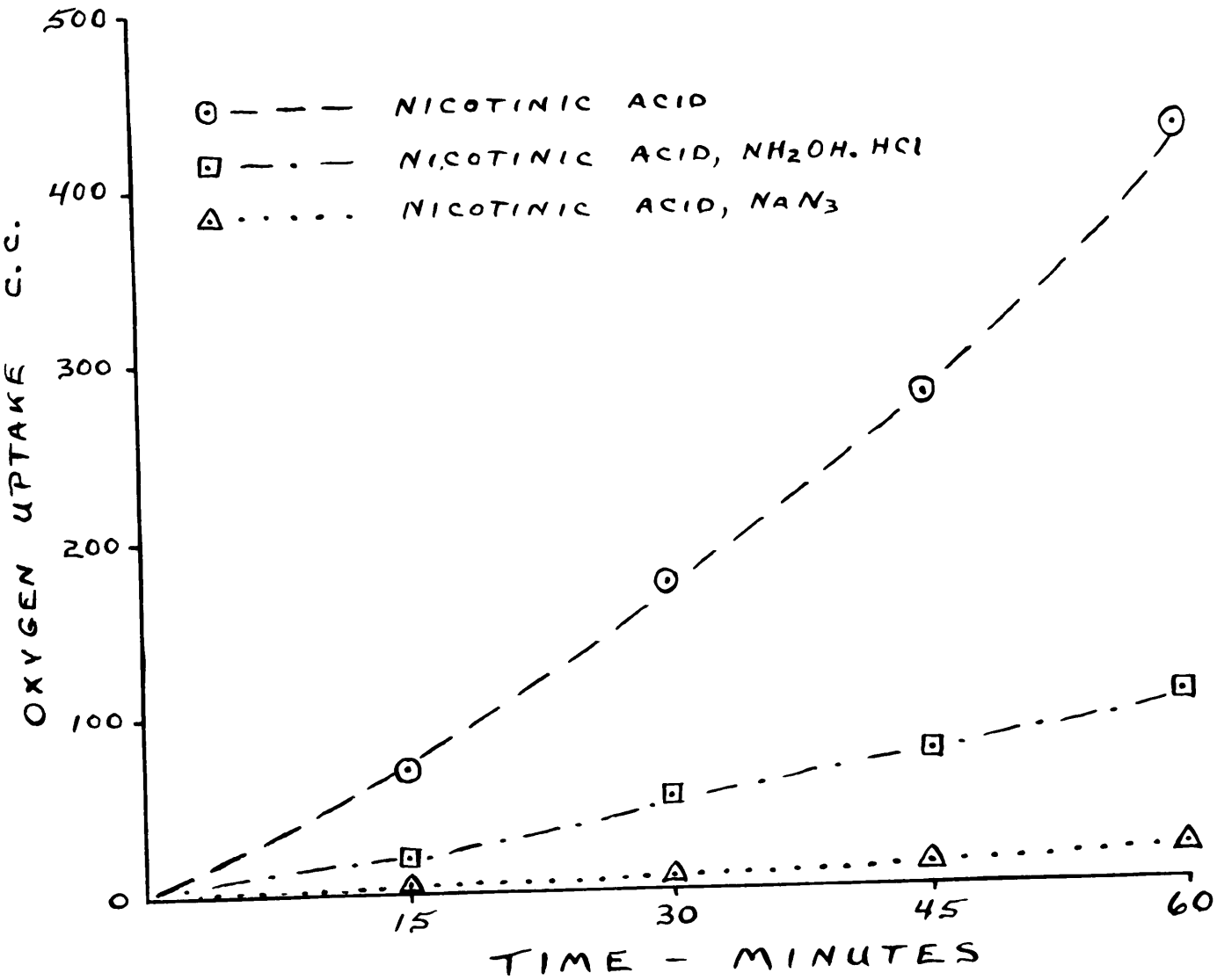

Fig. III - Oxygen uptake of Ps. fluorescens 30 with nicotinic acid as influenced by NaN_3 and $NH_2OH.HCl$.

In order to find the oxygen uptake per molecule of nicotinic acid small amounts of the substrate were added to the suspension and it was observed when cessation of oxygen consumption occurred. Four to five moles of oxygen were taken up per molecule of nicotinic

acid. To determine more exactly the oxygen uptake, trichloroacetic acid was added to destroy the activity of the organism. The experiment was not satisfactory since a lag in oxygen uptake followed the addition of the trichloroacetic acid. Therefore, further experiments are in progress to determine whether monochloroacetic or dichloroacetic acids can halt the oxygen uptake more abruptly. Such an observation was recently reported by Lang (58) in experiments with mouse liver.

11. Inhibition of enzyme activity

The inhibition of enzyme activity by surface active agents such as toluene and octyl alcohol and by poisons of heavy metal catalysts such as sodium azide and hydroxylamine hydrochloride was also studied by the colorimetric determination of nicotinic acid. Separate tests for the activity of the suspension were carried out in the presence of M/60 sodium azide, M/60 hydroxylamine hydrochloride, 0.1 ml. of toluene and 0.1 ml. of octyl alcohol.

The percentage inhibition of normal activity by the poisons compared closely by the Warburg technique and by the colorimetric determination of nicotinic acid as indicated in Table V. The activity of the enzyme was almost completely inhibited by sodium azide.

Table V - Effect of some inhibitors on normal activity

Inhibitor	Percentage inhibition (i) Warburg	(ii) Colorimetry
NaN_3 M/60	99.8	98.0
NH_2OH M/60	78.4	75.6
Octyl alcohol	-	68.0
Toluene	-	80.0

Toluene and octyl alcohol strongly inhibited the activity of the suspension (Table V). The observation that the enzyme activity was greatly inhibited when the bacterial cells were ruptured by alternate freezing and thawing and also by cell poisons such as toluene and octyl alcohol indicated that a large part of the active enzyme was bound to the cell structure.

SUMMARY

Several cultures of bacteria were compared for their ability to destroy nicotinic acid. A culture of Pseudomonas fluorescens was used as a source of an enzyme which rapidly decomposed nicotinic acid but did not attack nicotinamide appreciably. This enzyme was active at room temperatures and could be employed to determine nicotinic acid at the pH required for the color reaction.

Surface active agents such as toluene and octyl alcohol inhibited the destruction of nicotinic acid and repeated freezing and thawing of a cell suspension destroyed 75-80% of the normal activity of the enzyme. The enzyme mechanism appeared to be oxidative and its activity was reduced by poisons of heavy metal catalysts.

PART II

STUDIES ON THE CHEMICAL DETERMINATION OF NICOTINIC ACID

PART II HISTORICAL INTRODUCTION

Nicotinic acid, 3-pyridine carboxylic acid, was first prepared by Huber (37) in 1867 by the oxidation of nicotine. Seventy years later Elvehjem and coworkers (22, 23) found that nicotinic acid effectively cured blacktongue in dogs and could be isolated from anti-blacktongue liver extracts. In the same year Fouts and coworkers (29) reported the first successful treatment of human pellagra with nicotinamide. Accurate determination of this vitamin became of immediate importance.

The experimental animals that show typical nicotinic acid deficiency are the dog, pig and monkey. Measurement of the growth response of the dog has been used with success (25, 83) but other biological assays such as the chick test (51) have not proven as satisfactory (17). Rubenstein and Shekun (73) described a sensitive test for nicotinic acid based upon the development of newly-hatched larvae of the moth Galleria mellonella.

The essential nature of nicotinic acid for certain microorganisms made possible growth tests using a dysentery bacillus (20, 54), a colon-typhoid bacteria (46), Staphylococcus aureus (47, 48),

Shigella paradysenteriae (30, 39), and Leuconostoc mesenteroids (40). However, the method of Snell and Wright (78) using Lactobacillus arabinosus has been most widely applied. The amount of lactic acid produced when this organism is grown on a specified medium was proportional to the amount of nicotinic acid present. Modifications of the basal medium (8, 38, 55, 75) improved the response of Lactobacillus arabinosus and increased the sensitivity of the method. Although this microbiological procedure proved generally satisfactory (33) it was subject to variations in technique and to the presence of non-nicotinic acid growth stimulants in cereals (2, 14, 15, 34, 44).

The reaction between pyridine derivatives and 2,4-dinitrochlorobenzene, proposed by Vongerichten (87), was adapted to the colorimetric determination of nicotinic acid in foods by Karrer and Heller (42) and in urine by Vilter, Spies and Mathews (86). Daroga (19) used phosphomolybdic acid which combines with nicotinic acid, i.e. after treating the washed, redissolved precipitate with a reducing solution, the color produced was matched with a standard in a tintometer. Lingane and Davis (59) and Tompkins and Schmidt (85) applied polarographic techniques to the

determination of nicotiric acid. These two methods were of limited application.

The Konig reaction (52) in which the pyridine ring is opened with cyanogen bromide has been the basis of the most accurate chemical methods. Pyridoxine, the only other vitamin known to contain the pyridine ring, has a methyl group in the alpha position and does not react with cyanogen bromide. However, all alpha-unsubstituted pyridine compounds such as nicotinamide, nicotinuric acid, nicotinic acid N-diethylamide, beta-picoline, alpha-aminopyridine and nepecotic acid gave positive tests (88). Kringstad and Maass (46) and Bandier (5) reported that the presence of potassium dihydrogen phosphate reduced the interference from these related compounds.

In an extensive study of compounds interfering in the Konig reaction Hodicek (50) found that the solvents isobutyl alcohol, benzene, toluene and propyl alcohol produce a slight color which was probably due to impurities. Ashford and Clark (4) reported that acid hydrolysis of wheat samples produced furfural which reacted with the reagents to give a non-specific color. Melnick (62) reduced this interference by adjusting the hydrogen-ion concentration during the color reaction. The derivative trigonelline produced an interfering

color when hydrolyzed by alkali (64, 67, 70). Wang and Kodicek (89) used a weak alkaline hydrolysis to overcome this difficulty in the analysis of nicotinic acid in urine.

Nicotinic acid seldom occurs naturally in the free form and its biologically active derivatives must be hydrolyzed prior to the chemical determination (25). Coenzymes I and II are easily hydrolyzed (50) and treatment with weak acid was sufficient to convert nicotinamide to the free acid (13). Nicotinuric acid can be hydrolyzed by alkali but is fairly resistant to acid (70). The nicotinic acid derivatives in plant materials require 30 minutes heating at 100°C with 2N acid (34). The pigments and interfering substances produced during hydrolysis were eliminated by various techniques.

The effectiveness of precipitating agents depends upon the type of sample being analyzed. Tungstic acid was proposed by Swaminathan (81) and has been widely applied to the analysis of blood and urine (1, 45, 69, 80). In the analysis of tissues Kodicek (50) precipitated the pigments with acetone or alcohol. Other precipitating agents commonly used include barium hydroxide (27), zinc hydorxide (31), and lead hydroxide (18, 36, 70).

Swaminathan (81) and Melnick and Field (63) removed interfering pigments from urine with charcoal. Melnick, Oser and Siegel (66) reported that preferential adsorption on charcoal removed most of the pigment from cereal hydrolyzates without loss of nicotinic acid. However, Dann and Handler (18) and Waisman and Elvehjem (88) found this technique unsatisfactory because of incomplete decolorization. Perlzweig, Levy and Sarett (70) showed that charcoals remove varying amounts of nicotinic acid from pure aqueous solutions in a range pH 1 to 10 and since nicotinic acid can be adsorbed on filter paper special treatments must accompany filtration. Wang and Kodicek (89) found that variation in the quality of the charcoal was an additional source of error. A method for the adsorption of nicotinic acid by medicinal charcoal was suggested by Giri and Naganna (32) but other workers were unable to obtain reproducible results.

Oxidation of interfering pigments with 4% potassium permanganate yielded almost colorless extracts of urine which had been washed with isobutanol but the use of the solvent required a corrective factor (89). Brown, Thomas and Bina (13) found hydrogen peroxide efficient for decolorizing cereal extracts and reported two procedures for the removal of the excess peroxides (14).

Perlzweig, Levy and Sarett (70) showed that nicotinic acid could be quantitatively adsorbed and eluted from Lloyd's reagent. All pyridine derivatives were adsorbed on Lloyd's reagent below pH 1 and although a large proportion of the pigments were adsorbed and eluted, all but a trace of these could be removed by precipitation with lead hydroxide. This technique has been applied to the determination of nicotinic acid in animal tissues by Dann and Handler (18) and to cereal products by Hausman, Rosner and Cannon (36). Colorless blanks could be obtained by this method but Wang and Kodicek (89) and Bina, Thomas, and Brown (11) pointed out that Lloyd's reagent does not eliminate chromogens.

Other workers have attempted solvent extraction of the colored complex which nicotinic acid forms with the amine following the cyanogen bromide reaction. Swaminathan (81) suggested amyl alcohol. Ritsert (72) and Perlzweig and coworkers (70) used butanol but Waisman and Elvehjem (88) found these solvents unsatisfactory for color comparison. Arnold, Schreffler and Lipsius (3) reported that the colored complex formed with p-aminoacetophenone was selectively extractable with ethyl acetate. This technique has been applied successfully and Brown, Thomas and Bina (13)

stated that the interfering substances in cereal extracts were not extractable in ethyl acetate.

The technique of color development followed a consistent plan but varied widely in detail. When the cyanogen bromide reaction had reached completion an aromatic amine was added. Maximum color intensity was allowed to develop and readings were made in a photoelectric colorimeter at the wave length of optimum transmission. The numerous procedures recorded in the literature differed in detail because (i) the cyanogen bromide reaction was carried out under different specified conditions, (ii) several aromatic amines having different properties could be used successfully, (iii) blank corrections were made in several ways.

From an investigation of the reported chemical methods for nicotinic acid it was evident that wider application of a single procedure could only be accomplished by a specific determination. Very little had been done to improve the specificity. The use of adaptive enzymes for the determination of nicotinic acid in blood was suggested by Allinson (1). Studies were undertaken with a view to applying specific bacterial enzymes to the chemical determination of nicotinic acid in foods.

EXPERIMENTAL

1. Conditions for the cyanogen bromide reaction

Using 2 ml. of cyanogen bromide in comparable aliquots, Harris and Raymond (37) heated the reaction mixture at 80°C for 10 minutes; Kodicek (50) allowed the solution to stand for 5 minutes at this temperature; and more recently Wang and Kodicek (89) reported that optimum conditions were obtained by heating at 50 to 60° for 5 minutes. Terri and Shimer (82) obtained reproducible results with 5 ml. of CNBr in a total volume of 20 ml. and a reaction time of 30 minutes at room temperature. When 1 ml. of CNBr in a total volume of 7 ml. reacted with nicotinic acid at room temperature, Steele (79) found that after 15 minutes the color produced on the addition of metol did not increase significantly. The lack of an adequate basis for comparing the various conditions under which the Konig reaction has been carried out made necessary an investigation of this reaction with respect to (i) the amount of CNBr required, (ii) time of reaction, (iii) temperature of reaction and (iv) pH.

Kodicek (50) observed no change in color intensity when the amount of CNBr was varied from 0.5 ml. to 4 ml. in 15 ml. of solution. Provided that a reasonable excess was used, the amount of CNBr was not

important (35). One ml., which was shown to be adequate (79), was used in this study. Maximum color developed immediately when m-phenylenediamine was used. This amine was most applicable because the change in pH required to stabilize the color terminated the CNBr reaction.

One ml. of CNBr was allowed to react with 25 micrograms of nicotinic acid (5.5 ml. at pH 6.0) at different temperatures for given periods before color development (Table VI).

Table VI - Relation between time and temperature of CNBr reaction.

Time	Photometric Density 20°C	40°C	60°C
3 min.	.180	.301	.509
6 "	.272	.438	.608
9 "	.319	.561	.699
12 "	.387	.602	.688
15 "	.403	.648	.688
20 "	.469	.668	.678
25 "	.509	.688	.688
30 "	.553	.699	.678

The color was read in the Coleman Spectrophotometer at 420 millimicrons with the galvanometer scale reading adjusted to 100 with distilled water. The photometric density was calculated from the formula D=2-log G where D is the photometric density and G, the galvanometer reading in percent transmission.

The reaction was completed in 6 minutes at

60°C but at room temperature the reaction was still incomplete at the end of 30 minutes. Using 5 ml. CNBr at room temperature the color intensity increased steadily for 20 minutes but the greater dilution decreased the sensitivity.

The time required to complete the CNBr reaction was found to depend on the temperature and the amount of CNBr. The reaction must reach completion before the addition of the aromatic amine, since the resulting pH is no longer favorable.

The hydrogen-ion concentration at which the Konig reaction was carried out affected the intensity of the color and the optimum pH varied with different amines. Using p-aminoacetophenone, reproducible results were obtained within a range of pH 5.5 to 7.5 (80). Metol gave good results at pH 5.0 to 6.1 with the optimum at pH 5.8 (79). With orthoform the reaction was maintained within pH 6.2 to 7.0 (61).

Standard solutions containing 25 micrograms of nicotinic acid were adjusted over a range of pH 5.0 to 8.0 with phosphate buffers before the addition of the CNBr. The intensity of the color produced by m-phenylenediamine and aniline at the different hydrogen-ion concentrations is indicated in Table VII.

Table VII - The effect of pH on the color produced in the CNBr reaction.

pH	Photometric density	
	m-phenylenediamine	aniline
5.0	.668	.496
5.3	.638	.475
5.6	.594	.502
6.0	.545	.538
6.2	.523	.509
6.6.	.495	.456
7.0	.469	.398
7.4	.462	.377
8.0	.469	.398

Terri and Shimer (82) used a buffer at pH 6.6 with m-phenylenediamine but it was evident that a more intense color was produced at pH 5.0 Aniline appeared to give optimum results at about pH 6.0

To standardize the CNBr reaction a M/5 phosphate buffer (pH 6.0) was used in all further experiments. The adaptive enzymes were active at this pH so further adjustment was unnecessary.

2. Properties of some aromatic amines

When the pyridine ring has been opened by CNBr, the addition of certain aromatic amines produces a more intense color. Since color development depends on the presence of the amine group, several different amines have been proposed, such as aniline (50),

p-aminophenol (80), b-naphthylamine (27), metol (p-methyl-aminophenol sulfate)(7), p-aminoacetophenone (38), p-aminophenylsulfonamide (71), orthoform (methyl m-amino-p-hydroxybenzoate)(61), m-phenylenediamine (82) and procaine hydrochloride (18). The properties of these amines differ widely and a comparison based on the literature is complicated by variations in technique and by the fact that the readings have been made at different wave lengths.

Using standard solutions at pH 6.0, the color produced by several amines with 25 micrograms of nicotinic acid was compared (Table VIII). The photometric densities were corrected by a blank reading of the color due to the amine reagent.

Table VIII - Comparison of the colors produced by several amines.

Amine	Time required for maximum intensity	Photometric density	Optimum Transmission
m-phenylene-diamine	0.5 minutes	.587	420 millimicrons
aniline	5 "	.589	420 "
orthoform	5 "	.237	440 "
p-aminoaceto-phenone	30 "	.398	400 "
metol	60 "	.203	420 "

3. Stabilization of color by hydrochloric acid

The stable color given with orthoform reported by

Martinek et al (61) could not be reproduced. The color faded rapidly after reaching a maximum in 5 minutes but it was observed that HCl decreased the rate of fading. Stabilization of the color for 20 minutes was effected if 0.3 ml. of 10% HCl was added at the peak of color intensity (Table IX). All samples contained 50 micrograms of nicotinic acid and readings were made after adjusting the galvanometer to read 100 with distilled water.

Table IX - Hydrochloric acid as a stabilizer of the color given with orthoform.

Time after addition of orthoform	Photometric density			
	No HCl	0.1 ml 10% HCl	0.3 ml. 10% HCl	0.5 ml. 10% HCl
5 minutes	.414	.398	.347	.301
7 "	.352	.398	.337	.292
10 "	.268	.382	.337	.314
15 "	.229	.372	.332	.337
20 "	.221	.367	.332	.357
25 "	.214	.362	.332	.367
30 "	.211	.357	.347	.382

Aniline gave a color of good intensity but it faded quickly after reaching a maximum in 5 minutes. Stabilization of this color was attempted with HCl. The addition of 0.2 ml. of 10% HCl at the point of maximum color intensity prevented fading for 15 minutes (Table X). Each sample contained 50 micrograms of nicotinic acid. The

presence of HCl was observed to increase the color normally obtained with an equivalent aliquot.

Table X - Hydrochloric acid as a stabilizer of the color given with aniline.

Time after addition of aniline	Photometric density			
	No HCl	0.1 ml 10% HCl	0.2 ml. 10% HCl	0.3 ml. 10% HCl
5 minutes	.733	.886	.870	.854
7 "	.668	.870	.886	.870
10 "	.553	.824	.886	.870
15 "	.420	.770	.886	.854
20 "	.337	.699	.870	.824
25 "	.266	.620	.838	.810
30 "	.214	.569	.810	.796

Teeri and Shimer (82) were able to stabilize the color produced by m-phenlyenediamine for 20 minutes with HCl. It was necessary to determine the optimum amount of HCl to be employed with the volumes of solution used in the procedure adopted. 5 ml. aliquots of a standard solution of nicotinic acid (10 micrograms/ml.) and 0.5 ml. of M/5 phosphate buffer (pH 6.0) were heated at 60°C for 10 minutes with 1 ml. of CNBr. All tubes were cooled for 5 minutes in running tap water before the adding to each of 1 ml. of 5% m-phenylene-diamine. Varying amounts of HCl were added immediately and the stability of the colors was determined (Table XI). In the presence of 0.5 ml. of 20% HCl the color did not fade for 15 minutes, so this amount was used in all

further experiments.

Table XI - Hydrochloric acid as a stabilizer of the color given with m-phenylenediamine.

Time after addition of m-phenylene-diamine	Photometric density			
	No HCl	0.1 ml. 20% HCl	0.5 ml. 20% HCl	1.0 ml. 20% HCl
1 minute	1.000	1.000	.981	.745
3 "	.979	1.000	.981	.770
5 "	.939	1.000	.981	.782
10 "	.824	.939	.921	.810
15 "	.688	.838	.921	.810
20 "	.602	.782	.903	.770
25 "	.509	.732	.838	.745
30 "	.444	.699	.782	.721

4. Selection of an aromatic amine

The properties which determine the choice of an amine are (i) stability and intensity of the color, (ii) time required for color development, and (iii) the amount of color imparted to the blank by the amine reagent.

Metol has proven satisfactory because it gives a color which is stable for at least one hour. However, it was inconvenient in that one hour was required for the development of maximum color intensity and when compared with p-phenylenediamine or aniline, the color with metol was of low intensity.

Procedures employing p-aminoacetophenone require the addition of a specified amount of HCl. The addition of HCl to p-aminoacetophenone with or without CH$_3$OH, produced a color which was most intense at low concentrations

of HCl. Although this color faded at the concentration of HCl recommended in some procedures (89), this amine was considered to be unsatisfactory because it lacked specificity.

The color with orthoform was of low intensity. Although the addition of HCl gave satisfactory stabilization, the color intensity was less than was normally obtained.

Aniline gave a color of high intensity which could be stabilized by HCl. However, to reproduce the color with a given quantity of nicotinic acid the HCl had to be added at the exact point of maximum color intensity.

Although the reagent blank was comparatively highly colored, m-phenylenediamine was selected for use because a color of high intensity was developed immediately and it could be stabilized for 15 minutes by the prompt addition of HCl.

5. Optimum amount of amine

Dann and Handler (18) pointed out that the final color intensity depends on the concentration of the amine rather than the absolute quantity. Since high blank corrections decrease the sensitivity, the amount of the amine employed should be such as to give maximum color intensity without increasing the blank reading.

Following the completion of the CNBr reaction, varying amounts of 5% m-phenylenediamine were added to tubes each containing 25 micrograms of nicotinic acid. The color of the test solutions and the reagent blanks were measured (Table XII).

Table XII - Optimum amount of m-phenylenediamine

5% m-phenylene-diamine	Photometric density 25 micrograms nicotinic acid	Reagent blank	Difference
0.2 ml.	.367	.020	.347
0.5 "	.516	.039	.477
0.8 "	.602	.060	.542
1.0 "	.658	.081	.577
1.5 "	.688	.105	.583
2.0 "	.721	.134	.587

The difference in photometric density between the blank and the test solutions indicated that the color due to nicotinic acid increased up to 1 ml. of the amine reagent but with larger volumes the blank readings increased without a corresponding increase in the color attributable to nicotinic acid. In subsequent experiments 1 ml. of a 5% m-phenylenediamine was used. Similarly, it was found that 1 ml. of 4% aniline was the optimum amount.

6. The 'acid blank' correction

The discrepencies in the values reported for the nicotinic acid content of foods are due to variations in the methods employed to correct for the blank and to

different techniques. It is essential that the extracts be water-clear since the color resulting from the interaction of the reagents with residual pigments would be calculated as nicotinic acid. Apparently colorless extracts produced a slight color by reaction with the reagents.

The blank described by Bandier (5) consisted in adding all the reagents except the aromatic base to an aliquot of the test solution. However, CNBr reacts with nicotinic acid to produce a slight color even in the absence of the base (66). Other investigators omitted CNBr and added the aromatic amine to the blank tube. Here again, the base has been shown to react with substances in the hydrolyzates to produce a color similar to that given in the test for nicotinic acid (63). Melnick, Oser and Siegel (66) showed that this reaction did not occur in the presence of CNBr but concluded that the amine should not be added to the blank or low values will result. The nicotinic acid content of breads and flours was observed to vary depending upon the blank correction used in the calculations.

The separate measurement of the residual color in the extract and the color due to the reagents does not compensate for the side reactions known to take place in the test solution when the reagents are added to the

extract. Theoretically, a correct blank should contain all the reagents. Wang and Hodicek (89) found that CNBr did not react with nicotinic acid at low hydrogen-ion concentrations. The sample and all reagents could be included in one blank if HCl was added before the CNBr. This so-called 'acid blank' was adopted in this investigation. The same amount of 20% HCl (0.5 ml.) as was required to stabilize the color with m-phenylenediamine gave a satisfactory blank when added to the sample before the CNBr.

The acid blank was compared with the enzyme blank which is described below and with the more common correction secured by the separate measurement of the color given by the sample and reagent blanks (Table XIII). The color reactions were made with m-phenylenediamine.

7. The 'enzyme blank' correction

The bacterial suspension described in Part I was used as a specific reagent to destroy the nicotinic acid in an aliquot of a sample. The 'enzyme blank' was secured by applying the color reaction to this aliquot. Using two aliquots of an extract, the difference between the color developed by the test solution and the enzyme blank is a measure of the color produced by nicotinic acid. The color developed in the enzyme blank is an accurate measurement of non-nicotinic acid chromogenic

material.

Allinson added a heat-inactivated suspension of the bacteria to the aliquot used for full color development. No difference was found in the values obtained with and without the inactive suspension so this procedure was discarded.

The inactivity of each bacterial suspension was determined by the method outlined. The amount of suspension used to prepare the enzyme blank was sufficient to destroy 100 micrograms of nicotinic acid in 15 minutes. This amount of suspension (usually 0.1 ml.) was added to a 5 ml. aliquot of the water-clear extract plus 0.5 ml. of M/5 phosphate buffer, pH 6.0. After 30 minutes at room temperature the bacterial cells were removed by centrifuging at high speed for 15 minutes and 5 ml. of the clear supernatant were taken for the color reaction.

Table XIII - Comparison of three different methods of making the blank correction.

Sample	Photometric density		
	Reagent+sample	Acid blank	Enzyme blank
Standard sol'n 50 micrograms	.060	.063	.071
Yeast I	.069	.099	.108
" II	.079	.146	.167
" III	.079	.113	.119
Coffee	.116	.197	.292

Table XIII compares the enzyme blank, acid blank and the commonly used correction value representing the sum of the sample and reagent blanks. Only with

standard solutions was good agreement obtained with all three corrections. In the analysis of yeast which is known to have a relatively low content of interfering substances, a wide difference is evident between the ordinary blank and the acid blank. On the other hand, the acid blank compared favorably with the enzyme blank. With coffee which contains many interfering substances, the enzyme blank was much higher than the acid blank thus indicating that non-nicotinic acid chromogenic materials gave rise to color in the absence of acid which was not compensated for by the acid blank. In such cases, a correction based on the reagent and sample blanks would give erroneous values.

To ascertain if some substance in the coffee extract might have inhibited the enzyme activity, two enzyme blanks were prepared, to one of which was added 10 micrograms of a standard solution of nicotinic acid before adding the suspension. These two blanks were in close agreement indicating that enzyme activity as measured by destruction of nicotinic acid was not inhibited.

8. Interference from Lloyd's Reagent

A method of wide application for preparing water-clear extracts involves the use of Lloyd's reagent and lead nitrate. When m-phenylenediamine was added to

clear extracts prepared by this technique, a rapid increase in color intensity was observed if the addition of HCl to stabilize the color, was delayed. The color developed was independent of the cyanogen bromide reaction. Standard solutions which had not been treated with Lloyd's reagent did not give this color.

Two blank solutions were carried through the clarification procedure as outlined by Steele (79). At each step involving Lloyd's reagent, one was shaken vigorously in a stoppered 50 ml. centrifuge tube for one minute and the other for five minutes. Table XIV compares the color intensity developed by the addition of m-phenylenediamine to aliquots of these two solutions and shows the effect of delaying the addition of HCl.

Table XIV - Interference produced by Lloyd's reagent in blank solutions.

Sample	Photometric density HCl added immediately	HCl added after 5 min.
Distilled water	.071	.073
Solution I - shaken 1 min. with Lloyd's reagent	.086	.187
Solution II - shaken 5 min. with Lloyd's reagent	.097	.260

The color produced with m-phenylenediamine in blank extracts prepared with Lloyd's reagent was related to the time that the solution was shaken with Lloyd's

reagent and to the time that elapsed between the addition of the amine and the addition of the HCl. Standardizing the technique by shaking with Lloyd's reagent for 1 minute and adding the HCl immediately after adding the m-phenylenediamine yielded reproducible results.

The extracts were tested for lead with hydrogen sulfide which might have interfered but the result was negative. Folin and Berglund (28) called attention to the solubility of Lloyd's reagent in acid solutions. The Aluminon test indicated that the 0.2 N sulfuric acid employed in the adsorption of nicotinic acid dissolved a considerable quantity of aluminum. However, this ion was removed during the lead nitrate treatment and phosphate precipitation. An Aluminon test on the final extract was negative. The presence of inorganic salts of lead, aluminum or silica did not cause any apparent interference.

9. Adsorption of nicotinic acid on synthetic resins

The 'Duolite' synthetic resins used for the separation of amino acids are resistant to acids and bases (16, 26). Three of these resins were tested for their ability to remove nicotinic acid from solution.

Adsorption columns were prepared in Pyrex glass funnels of constant bore using ground resins which would pass through a 60 mesh but not through an 80 mesh screen. The resins were cyclized before use according to

the directions given by the manufacturer. Standard solutions of nicotinic acid (10 micrograms per ml.) were passed through the columns at different hydrogen-ion concentrations. The amount of nicotinic acid adsorbed was determined by colorimetric analysis. Anion exchanger A-2 and cation exchanger C-3 did not remove nicotinic acid from acidic, basic or neutral solutions. However, nicotinic acid was quantitatively removed from acid solution by cation exchanger C-1.

Columns were prepared containing 50 mgs. of resin C-1 and the amount of nicotinic acid adsorbed from standard solutions at different hydrogen-ion concentrations was determined by colorimetry (Table XV).

Table XV - Relation between hydrogen-ion concentration and nicotinic acid adsorbed by resin C-1.

pH	Nicotinic acid adsorbed
6.0	0 mgs.
3.0	0.75 "
1.0	2.0 "
0.2	0 "

100 micrograms of nicotinic acid in 5 ml. of 0.1 N HCl was quantitatively adsorbed and eluted by an equal colume of 0.4 N NaOH. Resin C-1 completely adsorbed the nicotinic acid from a 0.1 N sulfuric acid extract of yeast and it was eluted by 0.4 N NaOH. However, much of the color in the extract was also adsorbed and eluted along with the nicotinic acid. Shaking

a quantity of the resin with an acidified standard solution did not give complete adsorption of nicotinic acid. Nicotinic acid could not be separated from nicotinamide by resin C-1 since both appeared to be adsorbed with equal intensity.

10. Extraction of nicotinic acid

The concentration of the hydrolytic agent should be such as to ensure the complete extraction of nicotinic acid from the sample without the production or extraction of interfering substances. Acid hydrolysis is preferable since biologically active compounds are thereby converted to the free acid.

Nicotinic acid was completely extracted from animal tissues (50), yeast (34), and plant materials (62, 66) by treatment for one hour with boiling water but stronger hydrolysis may be required to extract related compounds. Strong acid or alkali hydrolysis can ensure complete extraction and conversion of nicotinic acid derivatives but this was shown to produce interfering substances when applied to cereals (13, 68). The chromogen produced by hydrolysis of cereal extracts with strong HCl gave a dark brown color with metol and a lemon yellow color with aniline and p-aminoacetophenone (13). Hausman, Rosner, and Cannon (36) compared HCl and H_2SO_4 in the hydrolysis of cereal products. The use of

HCl resulted in color not produced by H_2SO_4. Chemical methods do not compensate for the interfering substances produced in hydrolysis.

Determination of nicotinic acid in identical samples of yeast were made after hydrolysis for one hour at 100°C with different concentrations of acid. The color contributed was measured by the enzyme blank (Table XVI). Hydrochloric acid gave higher values for the enzyme blank and for total color. However, the color produced by nicotinic acid (total color minus enzyme blank) was constant and was independent of the extractant used. Extracting yeast with water gave values comparable to those by acid extraction.

Table XVI - Relation between enzyme blank and concentration of acid employed for hydrolysis.

Extractant	Photometric density		
	Enzyme blank	Total color	Difference
Water	.119	.420	.301
0.5 N H_2SO_4	.114	.409	.295
2.0 N H_2SO_4	.149	.432	.283
2.0 N HCl	.248	.538	.290

OUTLINE OF A PROPOSED METHOD FOR THE DETERMINATION OF NICOTINIC ACID

A sample of finely divided material (amounting to not more than 5 grams and containing 100 to 500 micrograms of nicotinic acid) is suspended in 75 ml. of 2N H_2SO_4 in a 100 ml. volumetric flask and placed in a boiling water bath for one hour with occasional agitation. The flask is then cooled to room temperature and the contents adjusted to pH 1.0 with 40% NaOH using methyl violet as an external indicator. The volume is made up to 100 ml. with distilled water and mixed thoroughly. Any suspended material may be removed by centrifuging. A 25 ml. aliquot is transferred to a 50 ml. centrifuge tube and one gram of Lloyd's reagent is added. A rubber stopper is placed in the tube and it is shaken vigorously for one minute. The tube is centrifuged and the clear supernatant is discarded. The Lloyd's reagent is washed twice by suspending in 10 ml. of 0.2 N H_2SO_4 and recentrifuging. The washings are discarded and the tube is inverted and allowed to drain well. After adding sufficient 0.4 N NaOH to make the total volume 25.5 ml., the contents are mixed thoroughly for one minute and centrifuged .

The clear supernatant is poured into a dry centrifuge tube containing 1.6 grams of powdered lead nitrate and the contents stirred with a glass rod. One drop of phenolphthalein is added to ascertain if the

solution is acid. An alkaline reaction usually indicates insufficient mixing and if the indicator is not decolorized by further stirring more lead nitrate is added until a slight excess is present. The tube is then centrifuged and the supernatant poured into a dry centrifuge tube. Solid Na_3PO_4 is added until the solution is pink to phenolphthalein and then the solution is adjusted to pH 6.0 with 10% H_3PO_4 using indicator papers. The precipitate is removed by centrifuging and the clear extract is decanted into a dry test-tube.

In each determination four 5 ml. aliquots of the extract are transferred to test tubes marked "a", "b", "c" and "d". The enzyme blank, "b", receives 0.5 ml. of M/5 phosphate buffer (pH 6.0) and 0.1 ml. of the bacterial suspension which is evenly dispersed by shaking. This volume of the suspension of Ps. fluorescens 30, prepared as described above, will destroy 100 micrograms of nicotinic acid in 15 minutes at room temperature. To each of the remaining tubes is added 0.6 ml. of the buffer. After standing for 30 minutes the cells are removed from "b" by centrifuging at 10,000 r.p.m. (using the six-place head for the International Multispeed attachment) for 15 minutes or longer. 5 ml. aliquots from each tube are transferred to clean test tubes. To the acid blank, "a", is added 0.5 ml. of 20% HCl. 1 ml. of a standard solution

containing 10 micrograms of nicotinic acid is added to "d" and 1 ml. portions of distilled water are added to tubes "a", "b" and "c". 1 ml. of CNBr is added to each tube and all are heated in a water bath at 60°C for 7 to 10 minutes. The tubes are cooled in a beaker of cold water 20°C for 5 minutes. 1 ml. of 1% m-phenylene-diamine is added to each of the four tubes and then 0.5 ml. of 20% HCl are immediately added to each tube with the exception of "a". The color is read within 15 minutes in the Coleman Spectrophotometer set at 440 millimicrons and with the galvanometer adjusted to read 100 against distilled water.

Since the factors affecting the color intensity may vary with different samples, the increment method of evaluation is used. The photometric density, log (I_o/I), can be calculated by the formula $D = 2 - \log G$, where D is the photometric density and G, the galvanometer reading in percent transmission.

"d" - "c" = the color developed from 10 micrograms of nicotinic acid.

"c" - "b" = the color due to nicotinic acid in the aliquot.

"b" - "a" = the color due to substances other than nicotinic acid.

$\frac{\text{"d" - "c"}}{10}$ = "d_1", the color produced by one microgram of nicotinic acid.

$\frac{\text{"c" - "b"}}{\text{"}d_1\text{"}} \times \frac{5.6}{5} \times \frac{100}{5} \times \frac{1}{\text{wt. of sample in gms.}}$ = micrograms of nicotinic acid per gram of the sample.

APPLICATION OF THE PROPOSED METHOD

Foods of plant and animal origin were analyzed by the proposed method and the results, compared with the values reported in the literature, are presented in Table XVII. Values calculated using the enzyme blank represent the nicotinic acid content of the sample. The difference between the results based upon the acid and enzyme blanks respectively represents the non-nicotinic acid chromogenic material.

The values based upon the enzyme blank were consistently lower than those calculated using the acid blank. The difference, however, varied with the sample being analyzed. Least interference was found in yeast while coffee and distillers' dried solubles contained the largest proportions of non-nicotinic acid chromogenic materials. The results obtained were in general agreement with the reported values.

Table XVII - Nicotinic acid content of foods analyzed by the proposed method.
(Results are expressed as milligrams per 100 grams)

Sample	Proposed Method		Reported Values	
	Enzyme Blank	Acid Blank	Chemical	Microbiological
Beef Liver, (raw)	14.6	16.6	9.2-25.0 *17.9 (60)	-
Ham, (cured, roasted)	5.30	6.36	-	2.80-4.42 (74)
Coffee, (roasted beans)	6.70	11.6	-	8.0-10.9 (83)
Brewers' Yeast, (dried, concentrated)	66.0	68.5	20.0-65.0(33) *40.0 (60)	23.0-60.0 (33) 60.0 (8)
Whole Wheat Bread	1.89	2.22	4.1 (15)	*2.88 (84) 3.80 (15)
Brown Bread	1.40	1.86	0.8-1.2 (60)	*1.80 (60)
White Bread (Canada Approved)	3.84	4.11	-	-
Distillers' Solubles (dried, wheat)	19.1	26.4	-	21.0-23.2 (9)

* Value generally accepted

SUMMARY

1. At room temperatures the cyanogen bromide reaction was incomplete after 30 minutes. Optimum conditions for this reaction were obtained by heating at 60°C for 6 minutes.
2. The color produced with orthoform, aniline and m-phenylenediamine was stabilized by hydrochloric acid.
3. Extracts prepared with Lloyd's reagent produced an interfering color with m-phenylenediamine. Treatment with Lloyd's reagent for 1 minute and the immediate addition of hydrochloric acid following the addition of the amine eliminated this interference.
4. Nicotinic acid was quantitatively adsorbed from acidified solutions by "Duolite" synthetic resin C-1 and was completely eluted with dilute alkali.
5. A bacterial suspension was used to destroy the nicotinic acid in an extract of the sample being analyzed. The 'enzyme blank' obtained by applying the color reaction to this extract measured the non-nicotinic acid chromogenic material.
6. A proposed method was applied to the determination of nicotinic acid in foods. The proportion of interfering substances varied with the material being analyzed.

CONCLUSION

A method is proposed for the determination of nicotinic acid in foods. A bacterial suspension which destroys the nicotinic acid in an extract of the sample is used to obtain an 'enzyme blank'. This provides a more adequate correction for the non-nicotinic acid chromogenic materials. The method was applied to several foods and the proportion of the interfering substances varied in the different materials analyzed.

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