THE STRUCTURE AND ESTIMATION OF NATURAL PRODUCTS FUNCTIONALLY RELATED TO NICOTINIC ACID

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The authors have attempted to list in this review most of the compounds chemically related to nicotinic acid and to indicate the biological activity of each compound. The importance of the active compounds in animal and bacterial metabolism is outlined. A summary is given of the chemical, physical biological, and microbiological methods available for the determination of nicotinic acid, nicotinamide, and coenzymes I and II. The application of these methods in nutritional and biological studies is discussed briefly.

Ten years ago little attention was given to the chemistry or biochemistry of nicotinic acid and related compounds. In 1934 nicotinamide was characterized as one of the hydrolysis products from coenzymes I and II, and in 1937 both the acid and the amide were shown to be active in the cure and prevention of canine blacktongue and human pellagra. These discoveries naturally stimulated a great deal of interest in the structure and estimation of these compounds in living matter. Much of the information is now fairly extensive, although more work is needed before a complete picture can be presented. It is the purpose of this review to summarize the most important developments during the past six years.

I. HISTORICAL

The first chemical production of nicotinic acid is credited to Huber. In 1867 he (58) obtained from nicotine, by oxidation with sulfuric acid and potassium dichromate, a compound which had the formula $C_6H_5NO_2$, but he did not recognize it as pyridinecarboxylic acid until 1870 (59). The term "nicotinic acid" was apparently used first by Weidel (158), who by the oxidation of nicotine with nitric acid produced a compound which he assumed to have the composition $C_{10}H_8N_2O_3$. Later Laiblin (86) showed that this compound was pyridinecarboxylic acid and that it was identical with the compound made by Huber. In 1879 Weidel (159) produced the same compound from β-picoline and thus demonstrated that nicotinic acid is β-pyridinecarboxylic acid.

Almost fifty years elapsed before nicotinic acid was isolated from natural products. Suzuki, Shimamura, and Odake (139) isolated the compound from rice polishings. Funk (47) isolated nicotinic acid from both yeast and rice polishings but found the acid itself to display no activity in curing pigeon beriberi. Vickery (144) isolated it from yeast without previous hydrolysis and suggested therefore that it was present in the free state.

In 1934 Warburg and Christian (154) isolated nicotinamide from coenzyme II and demonstrated its function as part of a hydrogen-transporting coenzyme (157). Shortly thereafter, Euler, Albers, and Schlenk (33) obtained nicotin-

amide from coenzyme I, and it was shown that both coenzymes were nicotinamide-adenine-dinucleotides, but that coenzyme II contained three molecules of phosphoric acid while cozymase or coenzyme I contained two. Kuhn and Vetter (84) also prepared nicotinamide from heart muscle.

These studies stimulated further interest in the possible nutritional significance of the acid and the amide. Williams (163) in 1917, impressed by the common occurrence of nicotinic acid and the antineuritic vitamin in several natural substances, again tested nicotinic acid and trigonelline, as well as other pyridine derivatives, for antineuritic potency but found that none of them caused any permanent improvement in polyneuritic fowls. Szymanska and Funk (141) attributed an appetite-stimulating and weight-preserving action to nicotinic acid and the amide. Again in 1937, Funk and Funk (48) found a larger food intake and better growth in rats and pigeons on certain diets when given the acid and especially the amide. Frost and Elvehjem (46) also observed a growth stimulus from nicotinic acid when fed with adenylic acid to rats on purified diets.

In 1936 Koehn and Elvehjem (72) prepared from liver a concentrate which was free of riboflavin and which was highly active in the cure of a chick dermatitis and canine blacktongue. Further purification gave concentrates which contained very small amounts of solid matter, and finally Elvehjem, Madden, Strong, and Woolley (31) demonstrated the activity of nicotinic acid in the cure of blacktongue and isolated nicotinamide from the concentrates. The activity of nicotinic acid in the treatment of blacktongue was soon verified by a number of workers (22, 126, 135). The first report of its successful use in human pellagra was made by Spies, Cooper, and Blankenhorn, and by Fouts in November 1937 (see reviews by Elvehjem (30) and Smith (128)). It is estimated that 400,000 pounds of nicotinic acid will be manufactured in 1943 for therapeutic use and for the fortification of foods.

In 1937 Knight (69) found that nicotinic acid was an essential growth factor for *Staphylococcus aureus* and that nicotinic acid was present in the active preparations of the *Staphylococcus* growth factor. Mueller (99) showed that nicotinic acid is essential for diphtheria bacillus, Koser, Dorfman, and Saunders (78) for dysentery bacillus, and Fildes (44) for *Proteus*. These studies established the importance of nicotinic acid in bacterial metabolism.

Structure and Properties

A. Nicotinic acid and compounds closely related to its metabolism

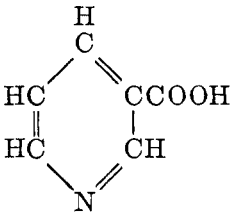
1. Nicotinic acid: $C_6H_5NO_2$; *molecular weight, 123; melting point, 235–237°C.*

Nicotinic acid occurs as white needle-like crystals or as a crystalline powder. It is soluble to the extent of 1 g. per 60 cc. of water or 80 cc. of alcohol at 25°C. and is freely soluble in hot water and hot alcohol. It is a comparatively weak acid: $pK_a = 4.76$. It is not destroyed by boiling acid or by alkali.

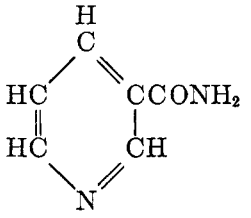
2. Nicotinamide: $C_6H_6N_2O$; *molecular weight, 122; melting point, 129–131°C.*

Nicotinamide is a white crystalline powder with a slightly acid taste. It is soluble at 20°C. in two parts of water, four parts of 86 per cent alcohol, eight

parts of glycerol, and twenty parts of acetone. It is slightly soluble in ether and insoluble in benzene. Its aqueous solution is slightly alkaline (pH 8.0). Nicotinamide is hydrolyzed to nicotinic acid on heating with strong alkali or acid.



Nicotinic acid



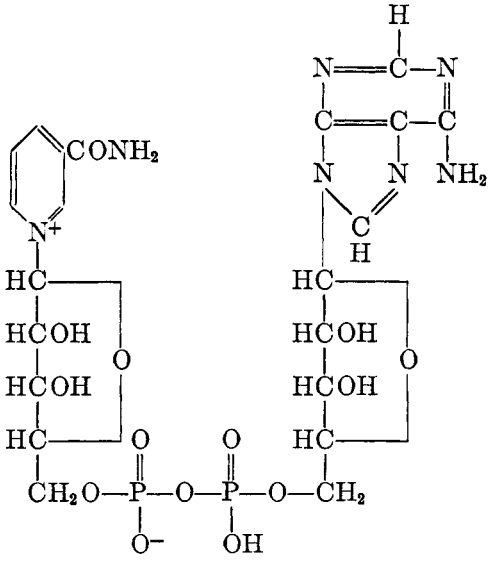
Nicotinamide

3. Coenzyme I: $C_{21}H_{27}N_7O_{14}P_2$; *molecular weight, 663*

This coenzyme (diphosphopyridine nucleotide; cozymase) is widely distributed in nature but it is generally prepared from yeast. Several different procedures have been used, and the details of these methods have been reviewed by Schlenk (119). About 0.5 g. of the pure compound is obtained from 10 kg. of yeast. The final product is not crystalline and probably not entirely pure.

In pure form cozymase is colorless and readily soluble in water. It titrates as a monobasic acid. The isoelectric point is at pH 3.1.

The following structure was proposed for cozymase by Schlenk and Euler (123) in 1936, and the final step in verifying this structure has recently been published by Schlenk (122). Adenine was demonstrated in the molecule by Euler and



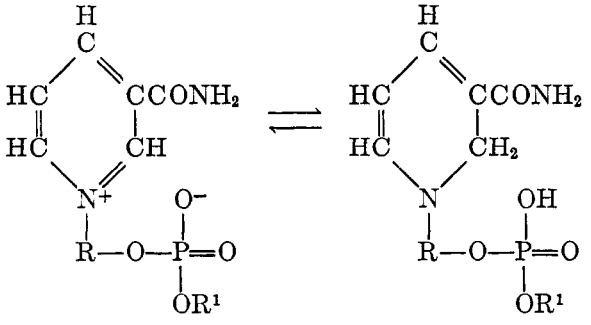
Coenzyme I; cozymase

Myrbäck (39), and later Warburg and Christian (155) and Euler, Albers, and Schlenk (34) showed cozymase to be a dinucleotide with one purine (adenine) and one pyridine (nicotinamide) nucleus. The carbohydrate appeared to be

pentose, since inosinic acid was obtained upon deamination. Schlenk (120) obtained 2 moles of pentosephosphoric acid per mole of cozymase and Euler, Karrer, and Becker (37) showed that the phosphoric acid is linked to the ribose in the 5-position. The existence of a pyrophosphate linkage in cozymase was established by the isolation of adenosinediphosphoric acid after alkali treatment.

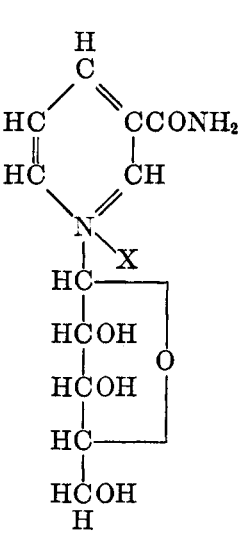
Another problem concerned the linkage between nicotinamide and the rest of the molecule. Warburg (155) showed that in the case of coenzyme II the nicotinamide reacts with two atoms of hydrogen in the presence of a suitable substrate and an active enzyme to form a dihydro compound. This reaction can also be produced with hydrosulfite in slightly alkaline medium. The oxidized form of cozymase has a maximum absorption of 260 mμ but after reduction the intensity at 260 mμ is somewhat reduced and a new maximum appears at 340 mμ. Thus the center of the coenzyme activity resided in the nicotinamide nucleus. The type of linkage between nicotinamide and the rest of the molecule was established by Karrer and his coworkers (67). It was found that nicotinamide iodomethylate showed the same light absorption, fluorescence, and color upon reduction and reoxidation as cozymase and coenzyme II showed. These results indicated that the nicotinamide is found as a quaternary pyridinium base. The free nicotinamide with tertiary nitrogen is not reduced by $Na_2S_2O_4$, in contrast to cozymase, the iodomethylate, and trigonelline, which is the methylbetaine of nicotinic acid. The preparation of nicotinamide derivatives containing carbohydrate radicals on the ring nitrogen gave compounds which showed properties more like those of the coenzyme (66). Tetraacetylglucosidonicotinamide bromide was prepared in pure form, but attempts to prepare the arabinose and xylose derivatives gave only oily products. The model nucleoside not only showed the same optical properties as the coenzyme but showed the same degree of stability to alkali.

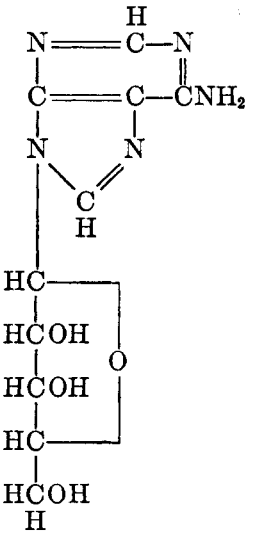
When the pyridinium model compounds are reduced to the dihydro compounds, the ring nitrogen of the reaction products become trivalent and they do not contain the acid group. The phosphoric acid in the coenzyme corresponds to the acid group in the model compounds. Upon reduction an acid group is liberated, as follows:



Finally Schlenk was able to obtain nicotinamide nucleoside from cozymase. Since treatment of the coenzyme with either acid or alkali breaks the linkage

between nicotinamide and the pentose, it was necessary to resort to the use of a specific phosphatase. The enzymatic hydrolysis gave the two nucleosides.





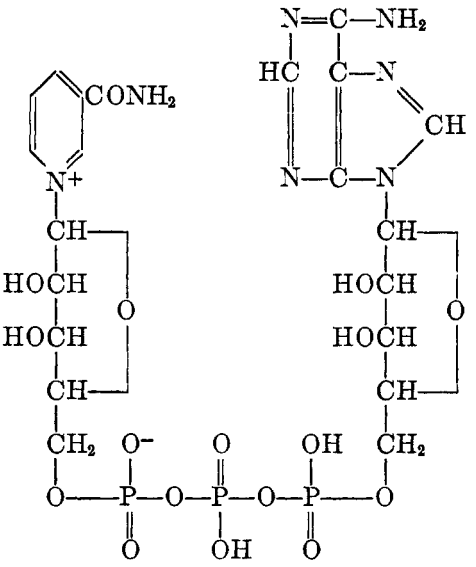
The nicotinamide nucleoside had properties very similar to those of the model compounds prepared by Karrer. Schlenk (122) and Euler, Karrer, and Usteri (38) have definitely proven that the carbohydrate group is ribose. Schlenk also confirmed the earlier investigations that showed that both of the ribosephosphoric acid molecules of cozymase have the phosphoric acid radical linked to carbon atom 5 of the pentoses.

The structure as proposed in 1936 is therefore clearly established; however, the compound has not been synthesized.

4. Coenzyme II: $C_{21}H_{28}N_7O_{17}P_3$; *molecular weight, 743*

Although coenzyme II (triphosphopyridine nucleotide) is as widely distributed in nature as coenzyme I, it is present in much lower concentrations. It was first isolated from red blood cells by Warburg and Christian (157). The centrifuged cells are cytolyzed with water and after removal of the proteins with acetone, the coenzyme is precipitated as the barium salt. It is then dissolved in methanol–hydrochloric acid and precipitated with ethyl acetate. Coenzyme II may also be obtained from yeast by working up the mother liquor remaining after precipitation of cozymase as the cuprous salt. It has not been obtained in crystalline form and all preparations are readily soluble in water.

In contrast to enzyme I, this compound contains a third phosphoric acid residue. Warburg (156) proposed a partial structural formula for coenzyme II in 1936, and Euler and Schlenk (41) suggested the following formula on the basis of what was known about cozymase.



Since adenosinetriphosphoric acid has not been isolated as a breakdown product, the formula is not final. It is suggested that the third phosphoric acid group is linked to the adenylic acid part of the molecule in the same manner as is yeast adenylic acid. Since Adler and Elliot (7) have shown that coenzyme I can be converted to coenzyme II, it appears that the location of the phosphoric acid is the only question which has not been settled.

Table 1 shows that the stability of the two coenzymes is very similar.

TABLE 1

Stability of coenzymes I and II

FORM OF ENZYME	TREATMENT	COENZYME I	COENZYME II
Oxidized form	0.1 *N* HCl at 100°C.	50 per cent destroyed after 8 min. (42)	50 per cent destroyed after 7.3 min. (154)
	0.1 *N* NaOH	50 per cent destroyed after 17 min. (20°C.) (42)	50 per cent destroyed after 12 min. (23°C.) (154)
Reduced form	0.1 *N* HCl at 20°C.	Activity disappears (42) immediately (106)	Activity disappears immediately (157)
	0.1 *N* NaOH at 100°C.	Slight decrease after 10 min. (8)	
	0.1 *N* NaOH at 20°C.	Stable (8)	Stable (157)

While the two enzymes have similar properties and almost identical structures they show great specificity in their functions. In most cases a dehydrogenase which requires coenzyme I for activity cannot function in the presence of coenzyme II. A list of dehydrogenases requiring coenzyme I and coenzyme II is given by Schlenk (119).

5. Trigonelline: $C_7H_7NO_2$, *molecular weight 137; melting point, 215–218°C.*

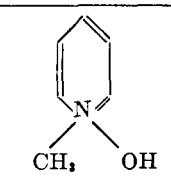
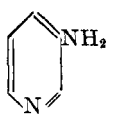
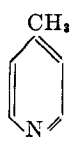
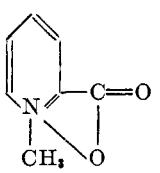
This compound was first isolated from plant material by Jahns (61) in 1885. In 1912 Ackermann (2) found that dogs given fairly large amounts of nicotinic acid excreted in the urine equal amounts of trigonelline and nicotinuric acid. It was isolated from starfish by Holtz, Kutscher, and Thielman (56) in 1924 and from liver extract by Subbarow and Dann (137).

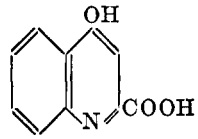
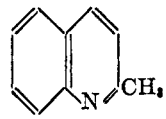
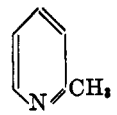
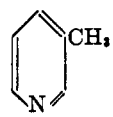
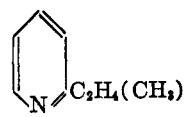
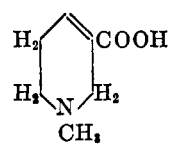
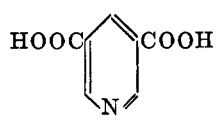
6. Nicotinuric acid (nicotinylglycine): $C_8H_8N_2O_3$, *melting point 240–242°C.*

This compound is a common constituent of urine but recent work by Sarett, Perlzweig, and Levy (117) indicates that in human urine the excretion of nicotinuric acid is very small in comparison to the amount of trigonelline. Nicotinuric acid is prepared by adding nicotinic acid chloride to a cold aqueous solution of glycine ethyl ester in slightly alkaline solution. The compound is crystallized from dilute hydrochloric acid solution. It is freely soluble in water and alcohol.

B. OTHER PYRIDINE COMPOUNDS FOUND IN NATURE

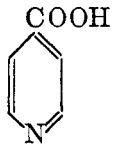
The following summary is taken from Bandier (16):

COMPOUND	ISOLATED FROM	FOUND BY
 Methylpyridinium hydroxide	Human urine	Kutscher and Lohmann (85) Ackermann and Kutscher (5) Ackermann *et al.* (3, 4)
 β-Aminopyridine	Liver extract	Subbarow, Dann, and Meilman (138)
 γ-Picoline	Horse urine	Achelis and Kutscher (1)
 Homarin	Lobster muscle; certain shell starfish	Hoppe-Seyler (57)

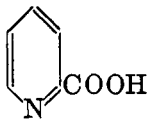
COMPOUND	ISOLATED FROM	FOUND BY
 Cynurenic acid	Dog urine	Liebig (89) and others
 α-Methylquinoline	Anal gland of the skunk	Aldrich and Jones (9)
 α-Picoline	Animal tar	Weidel (159)
 β-Picoline	Animal tar	Weidel (159)
 α-Methylethylpyridine	Animal tar	Weidel and Pick (161)
(C_7H_9N) Lutidine	Animal tar	Weidel and Herzig (160)
 Arecaidine	Naturally occurring alkaloid	Jahns (62)
 Dinicotinic acid	Liver extract	Subbarow (61)

C. OTHER COMPOUNDS RELATED TO NICOTINIC ACID

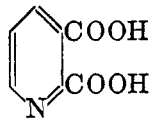
Other compounds related to nicotinic acid are listed below:



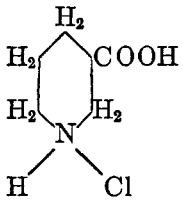
Isonicotinic acid
(m.p. 310–315°C.)



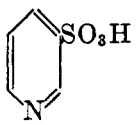
Picolinic acid
(m.p. 137°C.)



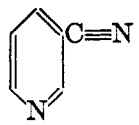
Quinolinic acid
(m.p. 190°C.)



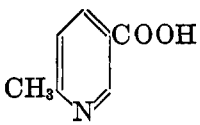
Nipecotic acid hydrochloride
(m.p. 237–239°C.)



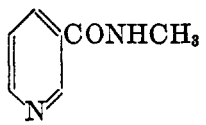
β-Pyridinesulfonic acid (decomposes)



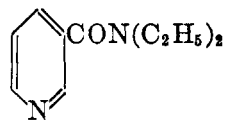
Nicotinonitrile
(m.p. 50°C.)



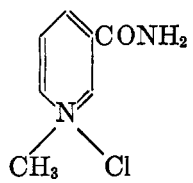
6-Methylnicotinic acid
(m.p. 206–207°C.)



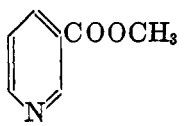
N-Methylnicotinamide
(m.p. 104–105°C.)



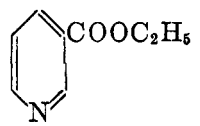
N,*N*-Diethylnicotinamide
(coramine)



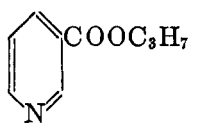
Nicotinamide methochloride
(m.p. 235°C.)



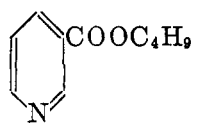
Methyl nicotinate



Ethyl nicotinate
(b.p. 86–88°C. at 5 mm.)



Propyl nicotinate
(b.p. 96–100°C. at 5 mm.)



Butyl nicotinate
(b.p. 111–117°C. at 5 mm.)

Since the activity of nicotinic acid was first demonstrated with the dog, it was natural that the studies on the biological activity of related compounds would

be made on this animal. In 1938 Woolley, Strong, Madden, and Elvehjem (164) tested about twenty different compounds on a semiquantitative basis. It was evident immediately that a rather specific structure is required for anti-blacktongue activity. The α- and γ-isomers of nicotinic acid (picolinic acid and isonicotinic acid) were found to be inactive. Hexahydronicotinic acid (nipecotic acid) was also inactive. All the compounds tested in which one of the ring hydrogens had been substituted by a methyl or a carboxyl group or in which a methyl group had been added to the ring were inactive. The replacement of the carboxyl group by a sulfonic acid group or by a cyano group or the removal of the carboxyl entirely (pyridine) led in each case to inactive compounds. All

TABLE 2

Compounds which have shown no biological activity

COMPOUND	DOG	DYSENTERY BACILLI	*Staphylococcus aureus*	MAN	*Lactobacillus arabinosus*	*Proteus*
Pyridine	− (164)*					
β-Acetylpyridine hydrochloride	− (164)	− (28)				
6-Methylnicotinic acid	− (164)	− (28)				
Nipecotic acid	− (164)	− (28)				
Nicotinonitrile	− (164)	− (28)	− (70)			
Isonicotinic acid	− (164)	− (28)	− (70, 88)			
β-Pyridinesulfonic acid	− (164)	− (28)				
Picolinic acid	− (164)	− (28)	− (70, 88)	− (132, 148)		− (108)
Trigonelline	− (164)	− (28)	− (70, 88)	− (131, 132, 148)	− (130)	− (108)
β-Aminopyridine	− (136, 137, 164)	− (28)		− (131, 132, 148)		
Nicotinamide methochloride	− (164)				− (80)	

* Literature references are given in parentheses.

these inactive compounds are listed in table 2, together with the organism on which tests have been made.

The studies with the dog indicated further that, in addition to nicotinic acid and its amide, only those compounds possess anti-blacktongue potency which are capable of oxidative or hydrolytic conversion to those substances in the body. Alkyl-substituted amides and the ethyl ester proved to be active. β-Picoline, which might be expected to be oxidized to nicotinic acid in the body, showed a fair degree of activity. Subbarow, Dann, and Meilman (138) reported that β-aminopyridine was highly active in the dog, but Strong, Madden, and Elvehjem (136) were unable to demonstrate any activity, and in a later note Subbarow and Dann (137) also reported the compound to be inactive. Later work has

shown it to be inactive in man (132, 148) and in bacteria (28). Woolley, Strong, Madden, and Elvehjem (164) reported that nicotinuric acid was active, but recently Dann and Handler (23) have presented results which lead them to believe that nicotinuric acid is not a blacktongue preventative but may be able partially to replace nicotinic acid in the diet of the dog. Sarett (115) also found that dogs maintained on a low intake of nicotinic acid are unable to hydrolyze nicotinuric acid, but excrete it completely without metabolic change. Sarett, Huff, and Perlzweig (116) found almost 100 per cent of the nicotinuric acid in the urine of humans after intravenous injection of 100-mg. doses. Dorfman (28) found nicotinuric acid to be active for dysentery bacilli. If bacteria can hydrolyze nicotinuric acid, it should be active upon oral administration in animals; however, the positive results may have been due to impurities of free nicotinic acid.

Many of the compounds found active in the dog were soon tested on humans. Spies, Bean, and Stone (131) found nicotinic acid, nicotinamide and sodium nicotinate active, *N*,*N*-diethylnicotinamide (coramine) somewhat active, and trigonelline inactive. Spies, Grant, and Huff (132) found picolinic acid, α-picoline, and β-aminopyridine somewhat toxic, but obtained some improvement with β-picoline, 2,6-dimethylpyridine-3,5-dicarboxylic acid, and dinicotinic acid.

There seems to be some question about quinolinic acid. Woolley, Strong, Madden, and Elvehjem reported it to be inactive in the dog, but Vilter and Spies (145) concluded that it cured pellagra in humans. Dann, Kohn, and Handler (25) studied the action of quinolinic acid in dogs more carefully and found that complete protection was obtained only when this compound was given at a level one hundred times that needed when nicotinic acid was used. Waisman, Mickelson, McKibbin, and Elvehjem (153) found quinolinic acid inactive when injected into blacktongue dogs at a level ten times the effective dose of nicotinic acid. Dorfman, Koser, Reames, Swingle, and Saunders (28) found it weakly active for dysentery bacilli. In 1939 Bills, McDonald, and Spies (18) reported that pyrazinemonocarboxylic acid and 2,3-pyrazinedicarboxylic acid promptly cured the glossitis of pellagra in humans. However, Dann, Kohn, and Handler (25) and Waisman, Mickelson, McKibbin, and Elvehjem (153) were unable to demonstrate any appreciable activity of these compounds in dogs. Smith (128) has recently concluded that the only compounds in addition to nicotinic acid which justify their use on patients with pellagra are nicotinamide and coramine.

Briggs *et al.* (19) found that the growing chick requires a dietary source of nicotinic acid for optimal growth and for prevention of chick "blacktongue" when purified rations are used. The minimal level required was found to be approximately 1.8 mg. per 100 g. of ration. Only a few compounds have been tested on the chick, but the results with the nicotinic acid esters are most interesting. Ethyl nicotinate is only one-half as active as the free acid, while the propyl and butyl esters show correspondingly greater potency. Apparently the esters of the higher alcohols are more easily hydrolyzed in the digestive tract of chicks. The dog seems to be able to utilize the ethyl ester as readily as the free acid. A summary of the results with animals is given in table 3.

In a recent review on the B vitamin requirements of different microörganisms, Peterson (110) reports that molds and yeasts do not require nicotinic acid but that thirteen different species of bacteria are dependent upon a supply of this growth factor. Koser and Wright (79) have reported very recently that a yeast (*Torula cremoris*) needs nicotinamide or the acid for prompt and abundant growth. The most extensive studies have been made with the dysentery bacilli. Dorfman, Koser, Reames, Swingle, and Saunders (28) tested twenty-four diffour different compounds on three strains of *Shigella paradysenteriae*. In general the results agree very well with those obtained on animals. They found the methyl, ethyl, propyl, and butyl esters active; in fact, the methyl ester was more active than the free acid. The results were also very similar to those obtained by Knight (70) and Landy (88), who used *Staphylococcus aureus*. There was some discrepancy in the case of coramine, since Dorfman *et al.* found it to be active, while Knight and Landy reported no activity for this compound. Most of the bacteria used the amide more readily than the acid. Dorfman found the amide ten times as potent as the acid when the development of the organisms was compared 24 hr. after inoculation, while Knight found the amide five times more active for the staphylococci. Mueller (100), however, found the amide only one-tenth as effective as the acid for diphtheria bacilli. (See tables 3 and 4.)

While most organisms requiring nicotinic acid can use either the acid or the amide, the *Haemophilus parainfluenzae* is a well-known exception. Lwoff and Lwoff (90) had been working on a growth factor (V factor) for this organism, and in 1937 they (91) showed that the V factor could be replaced by either coenzyme I or coenzyme II.

The requirement of the influenza organisms for the preformed cozymase, the well-known function of the cozymase molecule in isolated enzyme systems, and the occurrence of cozymase in living material have led to the belief that nicotinic acid or its amide are merely building blocks for the coenzymes. Experimental evidence indicates that this may not be true for all organisms. One might explain Mueller's (100) finding that nicotinic acid is more potent than the amide in the nutrition of the diphtheria organism in either of two ways: (*1*) owing to a peculiarity in the converting mechanism, the acid might be more readily converted to cozymase than the amide; (*2*) the cells may be more permeable to the acid than to the amide. But the report by Dorfman, Stewart, Horwitt, Berkman, and Saunders (29) that nicotinamide is more potent than coenzyme I or coenzyme II in promoting growth of the dysentery organism is a little more difficult to explain. Apparently the amide is performing a function *per se* or is being converted to a compound other than the coenzymes. However, differences in cell permeability may account for the different activities.

Schlenk and Gingrich (124) have recently reported the nicotinamide-ribose nucleoside to be active for the influenza organism. This organism's synthetic power apparently falls down in the conversion of nicotinamide to the nucleoside. Koser, Berkman, and Dorfman (77) report that certain *Pasteurellae* are able to synthesize cozymase from nicotinamide but not from nicotinic acid, while *Bacterium coli* is an example of those organisms which can synthesize the entire

coenzyme molecule. Fildes (44) has proved that *Proteus* is able to synthesize V factor from nicotinic acid, and we are perhaps safe in assuming that other organisms which grow on nicotinic acid are able to synthesize the coenzymes, if nicotinic acid is furnished.

Thus, it is evident that organisms vary greatly in their ability to synthesize the coenzymes and it appears that in the case of some organisms at least, cozymase may not be the most active compound.

TABLE 3

Comparative biological activity of nicotinic acid and related compounds

COMPOUND	MAN	DOG	CHICK (19)	DYSENTERY BACILLI (28)	*Staphylococcus aureus*	*Lactobacillus arabinosus*	*Proteus* (108)
Nicotinic acid	+++	+++	+++	+++	++	+++	+
Nicotinamide	+++ (131)*	+++ (164)		+++++	++++ (70, 88)	+++ (80, 130, 142)	+
Methyl nicotinate				++++	+++ (70)		
Ethyl nicotinate		++ (164)	+	++	++ (88)	− (80)	+
Propyl nicotinate			+	+		− (80)	
Butyl nicotinate			++			+ (80)	
Quinolinic acid	+ (145) − (25)	− (25, 153, 164)		+ (low)	− (88)		
β-Picoline	+ (132, 148)	+ (164)		−	− (70)		
Nicotinuric acid		+ (164) − (23)		+++	++ (88)	+++ (130)	+
N-Methylnicotinamide		++ (164)		++			
N,*N*-Diethylnicotinamide	++ (131)	++ (129, 164)		+	− (70, 88)		+

* Literature references are given in parentheses.

Studies involving sulfa drugs have been made both to obtain some clue to the mode of action of the drugs and to get a better understanding of nicotinic acid metabolism. McIlwain (94) has studied the bacteriostatic effects of sulfonic acid derivatives of nicotinic acid on *Staphylococcus aureus* and their counteraction by nicotinic acid, nicotinamide, and cozymase, and has found a definite competitive effect. West and Coburn (162) found that cozymase, but not nicotinic acid, reversed the bacteriostatic effect of sulfapyridine on this same organism. Straus, Dingle, and Finland (134) were unable to confirm these

results, but Spink, Vivino, and Mickelson (133) report cozymase to have counteracting effects under certain conditions. Two recent reports strengthen the belief that sulfapyridine interferes with nicotinamide–enzyme systems. Dorfman and Koser (27) have found that nicotinamide or cozymase reverses the depression of respiration in the dysentery organism caused by sulfapyridine. *p*-Aminobenzoic acid is unable to reverse this effect. Furthermore, the addition of nicotinamide to the medium decreases the amount of *p*-aminobenzoic acid necessary for counteraction of bacteriostatic effects. Teply, Axelrod and Elvehjem (143) have found nicotinamide, nicotinamide-ribose nucleoside, and cozymase to have almost equal activity in partially counteracting the bacterio-

TABLE 4

Comparative activity of nicotinic acid, nicotinamide, nicotinamide-ribose nucleoside, and cozymase

ORGANISM	NICOTINIC ACID	NICOTINAMIDE	NICOTINAMIDE-RIBOSE NUCLEOSIDE	COZYMASE
Bacterial growth promotion				
Influenza organism (124)	−	−	+	++
Pasteurellae (77)	−	+		+
Dysentery bacilli* (28, 29)	+	++		+
Staphylococcus aureus† (88, 100, 133)	+	++		++
Proteus (44)	+	+		+
Lactobacillus arabinosus (142)	+	+	+	+
Diphtheria organism (100)	++	+		
Sulfapyridine counteraction				
Staphylococcus aureus (growth) (133, 162)	−			+
Lactobacillus arabinosus (growth) (143)	−‡	+	+	+
Dysentery bacilli (respiration) (27)		+		+

* No direct comparison of nicotinic acid and cozymase is reported.

† No direct comparison of nicotinamide and cozymase is reported.

‡ Slight activity found was probably due to contamination with *p*-aminobenzoic acid.

static effect of sulfapyridine on *L. arabinosus*. Nicotinic acid was of such low potency that its effect might well be due to contamination with the highly active *p*-aminobenzoic acid. Further work involving more drugs and more organisms is necessary to clarify the picture, but there is little doubt that sulfa drugs can interfere with nicotinic acid metabolism.

The data summarized so far are important from a qualitative point of view, but in order to use these compounds successfully in nutrition and to understand the related metabolic processes more accurate quantitative methods must be used. Available methods for measuring the nicotinic acid content of natural materials include biological, physical, chemical, and microbiological procedures.

In the early studies the dog was the only animal that could be used for exten-

sive assays. At first a modified Goldberger diet was used (153) but more recently synthetic diets have been employed (118). In either case, the method is expensive and requires considerable time because a standard response must be established with pure nicotinic acid before the material to be assayed can be fed. The dog assay cannot be used for foods very low in the vitamin because sufficient food to give a typical response cannot be fed at one time; however, it still provides a means of checking the simpler methods. Liver seems to show a higher potency by the animal assay than by any of the other methods, a fact which may be due to the presence of so many other growth factors in liver. The chick has recently been used with considerable success in the assay of certain foods (19). The hamster has been suggested as a useful assay animal (113), but recent work indicates that this animal does not need nicotinic acid preformed in the diet (21). Biological assays measure total nicotinic acid activity and, as shown in table 3, different species may utilize different nicotinic acid derivatives with varying efficiency. It is thus difficult to decide which animal will give the best results in terms of human nutrition. It is unfortunate that so little quantitative work has been done with the monkey.

Methods involving the growth response of moth larvae (114), isolated tobacco roots (26), and pea roots (6) have been found to be of no practical value.

Spectrographic determination of nicotinic acid has been used by Holiday (55), and Karrer and Benz (64) developed a spectrophotometric procedure for nicotinamide.

Colorimetric methods for the estimation of nicotinic acid have been intensively investigated during the past six years. In 1899 Vongerichten (151) found that the addition of 2,4-dinitrochlorobenzene and alkali to pyridine produces a yellow color. Karrer and Keller (65) showed that nicotinic acid and the amide gave a similar reaction. Vilter, Spies, and Mathews (150) studied the reaction with a number of related compounds and concluded that pyridine, nicotinic acid, nicotinamide, *N*, *N*-diethylnicotinamide, α-picoline, and nicotine gave a color reaction, while trigonelline and picolinic acid yielded no color.

König (73) in 1904 showed that pyridine reacts with cyanogen bromide and a primary or secondary amine to give a color varying from yellow to violet. This reaction has been used most extensively for the chemical estimation of nicotinic acid and related compounds but a variety of amines have been used in the conjugation. Several workers (82, 95, 107, 127, 140) have used aniline. Bandier and Hald (17) found most consistent results with *p*-methylaminophenol and Harris and Raymond (53), Kodicek (71), and Arnold, Schreffler and Lipsius (11) based their procedures on the use of *p*-aminoacetophenone. Studies on the details of the reaction have been reviewed by Waisman and Elvehjem (152) and by Bandier (16). Bandier also includes extensive studies on the specificity of the color reaction. He found, using *p*-methylaminophenol, that pyridine yielded a yellow color which in equimolar amounts was more intense than the color with nicotinic acid. In the presence of potassium dihydrogen phosphate the color with pyridine was much less. Fortunately there is very little, if any, pyridine in most biological materials. Pyridine derivatives which

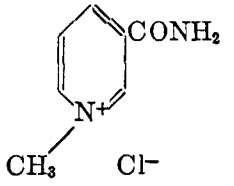
are substituted in the α- or γ-position do not give a color reaction. This fact probably explains why vitamin B_6 or pyridoxine gives no reaction. Kuhn and Löw (83) and Kringstad and Naess (82) had found that vitamin B_6 gave no color with cyanogen bromide and aniline. Pyridinium compounds also failed to give a color, and since the nitrogen atom in the coenzymes is pentavalent they do not show any activity until hydrolyzed. For equimolar quantities of pyridine derivatives substituted in the β-position the intensity of the color varied as follows: nicotinic acid 100, nicotinamide 142, nicotinuric acid 42, β-aminopyridine 15, nicotine 8, and *N*, *N*-diethylnicotinamide 6. β-Picoline yielded a very pale color. From these results it would appear that relatively little difficulty would be encountered from interfering substances in biological material except in the case of blood and urine from smokers.

The chemical procedure did give rather consistent results for animal tissues: for example, the following values were obtained for beef muscle in four different laboratories: Bandier (15) 4.9, Kringstad and Naess (82) 4.9, Kodicek (71) 4.3, and Waisman and Elvehjem (152) 4.8. The occurrence of interfering colors in natural materials such as bile pigments, riboflavin, carotenoids, etc., has proved a definite handicap in reading the color which is developed. Therefore, it is necessary either to remove the nicotinic acid from the pigments or to remove the pigments from the nicotinic acid before the color development. Melnick and Field (95) attempted to adsorb the pigments in an acid alcohol solution with a special charcoal. Dann and Handler (24) maintain that it is essential to remove all, or virtually all, of the color from the extract and have used Lloyd's reagent and lead hydroxide. They also showed that charcoal removes only part of the color and in addition removes nicotinic acid from the acid alcohol solution.

The chemical method has been used by several workers for the estimation of nicotinic acid in blood. Pearson (107) found the method described by Swaminathan (140) the most satisfactory for blood. Klein, Perlzweig, and Handler (68) have discussed the various methods proposed for blood and have outlined a procedure for the analysis of the blood cells and the plasma.

The estimation of nicotinic acid and related compounds in urine is important in order to carry out balance studies, but this determination is difficult not only because of the presence of interfering substances but because much of the ingested nicotinic acid is excreted as trigonelline. Rosenblum and Jolliffe (112) found the method of Bandier and Hald to be a simple and specific procedure for the estimation of nicotinic acid and amide in urine. More recent work has taken into account the presence of nicotinuric acid and trigonelline, and improved methods have been described by Melnick, Robinson, and Field (98) and by Perlzweig, Levy, and Sarett (109). The free nicotinic acid, the amide, and nicotinuric acid are determined after acid hydrolysis. This treatment does not affect the trigonelline, which is determined after alkaline hydrolysis. Measurements on trigonelline excretion are complicated by the fact that the compound is present in appreciable amounts in coffee and legumes and finds its way into the urine as well as that formed from ingested nicotinic acid. Sarett, Huff, and

Perlzweig (116) found the normal daily excretion of acid-hydrolyzable nicotinic acid derivatives in normal humans to be 0.7 to 2.2 mg. The daily excretion of trigonelline when the subjects were on a controlled diet was 9 to 13 mg. When high amounts of nicotinic acid are ingested, all of the vitamin cannot be accounted for in the urine. In this connection it is interesting to refer to the work of Najjar and Wood (104). They found in human urine a small amount of a substance soluble in butyl alcohol which gave a bluish fluorescence with ultraviolet light (substance F_2). When a dose of 50 mg. of nicotinic acid was given, an increased amount of the substance was excreted. An increase in the urine could be detected within an hour and persisted for 4 to 6 hr. They compared the fluorescence of the compound with that of twenty-seven different pyridine derivatives but concluded that none of the compounds tested could be identified with the unknown compound. Huff and Perlzweig (60) have recently concluded that F_2 appears to be *N*-methylnicotinamide or a labile precursor which yields the compound in the course of isolation.



Najjar, Scott, and Holt (103) have commented on this conclusion as follows: F_2 appears to be a pyridine compound. The original compound does not give a cyanogen bromide reaction but after alkaline hydrolysis a positive cyanogen bromide test is obtained. It is biologically active. Results indicate that one of the *N*-methyldihydronicotinamides is indistinguishable from F_2 by its adsorption properties, solubility in eleven organic solvents, and its reactions with alkali, potassium ferricyanide, nitrous acid, acetone, and sulfanilic acid. They do not feel justified in identifying F_2 as an *N*-methyldihydronicotinamide for three reasons: (*1*) because one of the *N*-ethyl isomers likewise possesses these identical properties; (*2*) because acetylation of F_2 and of *N*-methyl- and *N*-ethyldihydronicotinamides gives compounds with different fluorescent properties and solubilities; and (*3*) because the absorption of F_2 shows characteristic differences. It is obvious that we need to know more about these compounds before completely quantitative measurements can be made on urine.

Noll and Jensen (105) have discussed some of the difficulties encountered in applying the chemical methods to milk and milk products.

When the cyanogen bromide method was applied to plant materials (71, 152), values for nicotinic acid were obtained which were not reconcilable with the known fact that many cereals are low in the anti-pellagra factor. The direct extraction of cereals with alkali or acid results in the extraction of chromogens which give an exceptionally high final value. Kodicek (71), Waisman and Elvehjem (152), and Melnick, Oser, and Siegel (97) have obtained values more in accord with accepted nicotinic acid potency when an aqueous extract is made

first and then treated with acid or alkali. Hale, Davis, and Baldwin (49) have used the chemical procedure on a large variety of plant products and have compared the results with those obtained by microbiological assay. They conclude that plant materials may be divided into two classes on the basis of their response to various extraction and hydrolytic treatments. Alkaline hydrolysis of an aqueous extract gives the most satisfactory results for the non-chlorophyll-containing seed and roots, while acid hydrolysis of the chlorophyll-containing part of the plant gives the lowest and most accepted values. In general, there was excellent agreement between the chemical and microbiological results except in the case of certain forage material. They suggest that the chemical method must be used with caution, if at all, for the forage part of the plant.

It is also interesting to point out that Lamb (87) and Melnick and Oser (96) have used the chemical method for the quantitative estimation of nicotinic acid and nicotinamide when they occur together. This method is applicable largely to vitamin mixtures. When cyanogen bromide and aniline are used, the relationship between the maximum extinction coefficients and the time for their development is characteristic for the acid and the amide.

A number of microbiological methods have been proposed for the estimation of nicotinic acid. In 1938, Lwoff and Querido (92) described the use of *Proteus* and, in the same year, Fraser, Topping, and Sebrell (45) developed an assay based on *Shigella paradysenteriae* (Sonne) and Koser, Dorfman, and Saunders (78) used the dysentery bacillus. However, it was not until 1941 that a satisfactory procedure for assaying all types of materials was developed. Snell and Wright (130) used *L. arabinosus* as the test organism and developed a medium which has been highly successful. Krehl, Strong, and Elvehjem (81) have recently made slight modifications in the procedure. A great variety of biological materials have been analyzed by this method and at present there is no reason to doubt the accuracy of any of the results.

Best results are probably obtained with food materials if all the nicotinic acid-containing compounds are hydrolyzed to the free acid before the bacterial assay is carried out. However, the nicotinamide, the nucleoside, and coenzyme I all give equivalent activity with *L. arabinosus*. There may be some question about nicotinuric acid. Snell and Wright (130) concluded that it was active, but since it apparently is difficult to prepare nicotinuric acid free from nicotinic acid a final decision may have to be made later. Certain natural materials contain a compound which shows no activity in the *L. arabinosus* assay until it is hydrolyzed. Andrews, Boyd, and Gortner (10), working with cereals and cereal products, found that water and dilute acid extraction gave lower values for nicotinic acid than stronger acids and alkali. They concluded that this discrepancy could be attributed either to the formation of growth-stimulating substances or to the liberation of active nicotinic acid compounds by hydrolysis of a less active or inactive precursor. Evidence was presented which indicated that the second possibility was the correct explanation. Cheldelin and Williams (20) also found that acid or alkaline extraction of cereals gave higher

values than enzymatic digestion alone. The increase in both the acid and alkaline extractions was attributed to substances convertible to nicotinic acid, and it was suggested that trigonelline might be responsible for part of the increase in the alkaline treatment. They concluded that the substance formed by hydrolysis was actually nicotinic acid because of the similar results with both microbiological and chemical methods. Extensive work in the authors' laboratory also strongly suggests that the precursor not only yields nicotinic acid but that it is biologically active at least in the dog before hydrolysis.

One would expect that the quantitative estimation of coenzymes I and II would be more difficult because the molecular structure is more complicated and the molecule itself is easily disrupted. In spite of these difficulties, some rather accurate determinations have been made. Again, biological, physical, chemical, and microbiological methods have been used.

Harden (52) measured cozymase activity by using a press extract of yeast as a source of the enzyme systems and a boiled yeast extract as a source of the coenzyme. The method of the determination of coenzyme I as developed by Euler (32) and Myrbäck (101) is based on the principle that the addition of varying amounts of the coenzyme to a washed yeast preparation will produce rates of fermentation which are proportional, within certain limits, to the amount of coenzyme I added. The use of this yeast fermentation method has been described in detail by Axelrod and Elvehjem (12). Schlenk and Vowles (125) have also described improvements in the yeast fermentation method. Jandorf, Klemperer, and Hastings (63) have made use of the activity of coenzyme I in a glycolysis system rather than in fermentation. The amount of phosphoglyceric acid produced in a given time in the presence of bicarbonate buffer is measured monometrically in a Warburg apparatus. Very few measurements have been made in the case of coenzyme II, but the original system described by Warburg (157) may be used. If the coenzyme is the limiting factor in this system, the rate of oxygen uptake is a measure of its concentration.

Physical methods depend upon the spectrophotometric determination of the corresponding dihydro compounds. In this case the solutions containing the coenzymes must be free from impurities that absorb in the ultraviolet region in which the dihydro coenzymes exhibit their characteristic absorption. The details of these methods have been summarized by Hogness and Potter (54). This procedure has been used mostly in studies on the related enzyme systems rather than in studies on the distribution of the coenzymes in tissues.

A chemical estimation of the two pyridine nucleotides may be made by determining the amount of nicotinamide present in a tissue before and after hydrolysis. The increase in the amount of the amide can then be used to calculate the amount of coenzyme originally present. Euler and coworkers (43) used this method in their early studies on the distribution of coenzymes in dog and rat tissues.

The microbiological methods are based on the fact that *Hemophilus influenzae* and *Hemophilus parainfluenzae* require the preformed coenzymes.

In 1938 Kohn (74) developed the so called V factor technique for the estima-

tion of total coenzyme I and II, using *H. parainfluenzae* as the test organism. Vilter, Vilter, and Spies (146) developed a method very similar in nature but used *H. influenzae* as the organism. Further details of this method have been published by Vilter, Koch, and Spies (149). It is important to remember that these methods do not distinguish between the two coenzymes and also that Schlenk and Gingrich (124) have shown that the nicotinamide nucleoside is also measured if present.

Regardless of how the actual estimations are made, two difficulties are always encountered which affect the accuracy of the final values. Since cozymase has not been obtained in absolutely pure form, it is difficult to secure a standard for use in the various methods. Some of the values in the literature are probably high because the standard cozymase used had an activity considerably below 100 per cent. Recent studies by Handler and Dann (50) indicate that some of the preparations that have been used have a maximum absolute purity of 76 per cent.

The other problem involves the prevention of destruction of the coenzyme during the preparation of the sample for analysis. Euler and coworkers (35, 36, 40) observed very early that the coenzyme content of tissues decreases very rapidly after the death of the animal, especially when the cell structure is destroyed. Mann and Quastel (93) found that brain suspensions rapidly destroyed cozymase and suggested the presence of a nucleotidase. Liver and kidney were less active and skeletal and heart muscles contained little if any of the enzyme. They found that nicotinamide reduced the rate of destruction and postulated that the nicotinamide and cozymase compete for the active center of the nucleotidase. Handler and Klein (51) have studied the inactivation of the pyridine nucleotides by a number of different animal tissues and found that in each case nicotinamide is liberated from the rest of the molecule. Nicotinamide was found to be quite specific in preventing the decomposition. In quantitative methods, this destruction can be prevented by freezing the tissues as soon as they are removed from the animal (12).

In spite of the difficulties which have been discussed, rather definite values are available for the distribution of cozymase in a variety of biological materials. The cozymase content of liver falls between 400 and 800 γ per gram of fresh tissue. The amount probably varies with the level of nicotinic acid in the ration. Briggs *et al.* (19) found much more cozymase in the breast muscle of chicks when the diet contained 10 mg. per 100 g. than when the amount in the diet was less than 2 mg. per 100 g. but adequate to give normal growth. Axelrod and Elvehjem (13), as well as Kohn, Klein, and Dann (76), have shown a decrease in the cozymase content of liver and muscle, but little change in the brain or kidney during nicotinic acid deficiency in dogs and pigs. A definite decrease was found in the muscle from human cases of pellagra (14). The coenzyme concentration in the red blood cells of pellagrous and normal individuals has been studied by Kohn (75, 76), Spies (147) and Elvehjem (14). All agree that the coenzyme content may be increased by ingestion of nicotinic acid and that the decrease during nicotinic acid deficiency is only slight. It has

been concluded that there is no diagnostic value to be obtained from determinations of coenzyme I in borderline cases of deficiency diseases.

Pitman and Fraser (111) measured the coenzyme (V factor) content of urine from normal and deficient dogs but found no difference in the amount excreted.

The evolution of our knowledge of the biochemistry of nicotinic acid has followed much the same course as that for other biologically active substances. When the importance of nicotinic acid was first recognized the picture appeared to be very simple: namely, all living tissues contain coenzymes I and II and nicotinic acid is needed for the construction of these molecules. Certain species can manufacture the nicotinic acid needed for these compounds, while others must rely upon an outside source. In the case of humans, nicotinic acid must be supplied preformed in the diet and the syndrome resulting from an inadequate intake has come to be known as pellagra. As the studies continue it is still evident that living cells require highly specific structures but these structures are found in a variety of natural compounds, some of which are biologically active and others inactive. All these related compounds must be considered, whether we are measuring dietary intake, metabolism, or excretion, and the complete picture cannot be finished until they have been recognized and methods for their quantitative estimation established.

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