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Quantitative Response of the Dysentery Bacillus to Nicotinamide and Related Compounds.*

ALBERT DORFMAN, STEWART A. KOSER, M. K. HORWITT, SAM BERKMAN AND FELIX SAUNDERS.

From the Departments of Bacteriology and Parasitology and of Biochemistry, University of Chicago, and the Elgin State Hospital, Elgin, Illinois.

In a previous report we showed that many strains of dysentery bacilli require nicotinamide or certain closely related compounds for growth.[1] We have also studied the growth-promoting activity of a number of compounds structurally related to nicotinamide.[2, 3] It is the purpose of this paper to report certain quantitative studies on the activity of nicotinic acid, nicotinamide, coenzyme I and coenzyme II. These studies were carried out in an attempt to learn the mechanism of action of these compounds in promoting growth of dysentery bacilli. The results were also used for the development of a method for the estimation of nicotinamide and related compounds in blood.

It has been generally assumed that nicotinic acid is converted to nicotinamide and that the latter is converted to one or both of the pyridine-containing coenzymes. We have presented evidence for the conversion of the acid to the amide, based on the relative activity of the two substances in promoting growth of dysentery bacilli.[3] It was found that when the acid is used as a growth-promoting substance there is a very definite lag in growth as compared with that obtained when the amide is used.

If nicotinamide is converted to one of the known pyridine-nucleotides, one or both of them must have a growth-promoting activity greater than or equal to that of an equivalent amount of nicotinamide.

In order to determine the relative activities of the various substances the titration method previously described by us was employed.†[3] Figure 1 indicates the relative activity of nicotinamide,

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1 Koser, S. A., Dorfman, A., and Saunders, F., PROC. SOC. EXP. BIOL. AND MED., 1937, **38**, 311.

2 Dorfman, A., Koser, S. A., and Saunders, F., *J. Am. Chem. Soc.*, 1938, **60**, 2004.

3 Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Inf. Dis.*, 1939, **65**, 163.

† We are indebted to Professor Otto Warburg for a sample of pure coenzyme II and to Dr. A. Axelrod for a sample of pure coenzyme I prepared in Euler's laboratory.

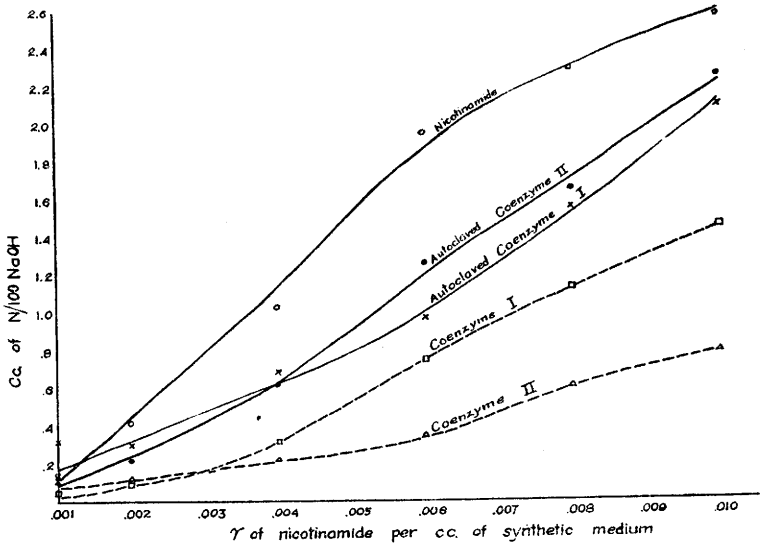

FIG. 1.

coenzyme I, coenzyme II, and autoclaved coenzymes I and II. It will be noted that nicotinamide is the most active growth-promoting substance, and coenzyme I is somewhat more active than coenzyme II. After the coenzymes are autoclaved (20 pounds, 30 minutes) the activity of each is markedly increased. Under certain conditions of heating it is possible to bring the activity up to that of an equivalent amount of nicotinamide. The results discussed above are obtained when the culture is titrated at the end of 4 days' incubation. If a shorter period is used the results are more striking since the coenzymes exhibit a lag period similar to that reported by us for the acid.

These findings are incompatible with the theory that nicotinamide functions solely as a building block for coenzyme I or coenzyme II. If nicotinamide is more active than an equivalent amount of either of the pyridine-containing coenzymes, it must have some importance other than, or in addition to, being a component of the known coenzymes. Metabolic experiments have confirmed this hypothesis although the details of this new mechanism are not yet known.[4]

Because of the rapid accumulation of evidence for the great importance of nicotinamide and related compounds in the nutrition and metabolism of various living forms there have been numerous attempts to develop methods for the determination of these compounds in biological materials.

[4] Dorfman, A., Koser, S. A., and Saunders, F., *Science*, 1939, **90**, 544.

The method which we have used is essentially the same as that described by us in a previous publication.[3] It consists in determining the amount of acid produced by a dysentery culture to which a definite dilution of a blood filtrate is added. The nicotinamide equivalent is determined by comparison with a standard curve (Fig. 1). The dilutions of the blood sample were made so that a minimum of 0.2 cc and a maximum of 0.4 cc was added to each tube containing 4.5 cc of basal medium. The final volume was made up to 5.0 cc. The tubes were incubated for 4 days at 37° and were then titrated with 0.01 *N* NaOH to a standard color of brom thymol blue. All blood samples were run at 2 different dilutions. Blank determinations were made on both inoculated and uninoculated controls. A series of nicotinamide standards was run with each set of blood samples. All determinations were run in triplicate.

The addition of a mixture of all of the available other known growth substances resulted in no increase in acidity, thus indicating that this test is specific for nicotinamide and related substances.[3] Schmelkes[5] has reported that thiazole-5-carboxylic acid is able to substitute for nicotinamide in promoting growth of dysentery bacilli. We have found that the activity of this compound is approximately one-hundred-thousandth that of nicotinamide, and therefore even if it did occur naturally it would not interfere with this assay.[6]

We first attempted to prepare our samples for assay by means of an acetone extract. It was found, however, that blood samples prepared in this manner gave results which were lower than those obtained with a water extract. Euler[7] has used an acetone method to determine the amount of free nicotinamide in blood. He obtained values of about 1 γ per cc when he used the cyanogen bromide method for determination of the nicotinamide. Our values when we used acetone for the preparation of the samples were somewhat higher, the normals averaging about 3 γ per cc. This difference is probably due to the fact that we employed 3 volumes of acetone, while Euler used 10 volumes. The latter quantity would extract less of the coenzymes.

The method used in most of our studies consisted in adding one volume of whole blood to 3 volumes of water within 10 minutes after the samples were drawn. This mixture was then heated to 70° for about 10 minutes and the coagulum filtered off. The preliminary heating is necessary to destroy the enzymes in blood which hydrolyze the coenzymes. The supernatant liquid is then ready for assay.

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5 Schmelkes, F. C., *Science*, 1939, **90**, 113.

6 Koser, S. A., Dorfman, A., and Saunders, F., PROC. SOC. EXP. BIOL. AND MED., 1940, **43**, 391.

7 Euler, H. V., and Schlenk, F., *Klin. Woch.*, 1939, **18**, 1109.

TABLE I.
Effect of Autoclaving on Amount of Nicotinamide Found in a Sample of Human Blood.

Determination No.	Dilution of filtrate, cc	N. amide added per cc, γ	Method of sterilization	Value found, γ per cc
1	.020	—	Filtered	6.1
	.030			6.2
2	.025	5	"	10.3
	.015			11.2
3	.025	—	Autoclaved	7.5
	.015			8.4
4	.015	5	"	13.2
	.010			12.0

Table I illustrates the results obtained on a normal sample of blood. The value obtained for total activity expressed as nicotinamide is dependent upon the method of sterilization employed. In this experiment the same blood was employed for all analyses. It will be noted that the activity is markedly increased by heat treatment. More drastic treatment resulted in little increase in activity. This increase in activity is to be expected on the basis of the results reported in the first part of this paper, since it is known that most of the nicotinamide present in blood exists in the combined form.

Table I also illustrates the type of results that can be obtained by running the samples at different dilutions, and the recovery of nicotinamide when added to the whole blood. We have occasionally experienced difficulties in obtaining checks at different dilutions. The explanation for these difficulties was not found.

Kohn[8] has pointed out that the amount of "V" substance present in human blood is a function of the hematocrit. In an earlier report we showed that all of the activity for the dysentery bacilli resides in the erythrocytes.[9] Table II shows the results obtained with 5 different bloods both before and after autoclaving, together with hematocrit values. The number of samples analyzed by us is insufficient to permit drawing any definite conclusions concerning the relation between hematocrit and nicotinamide.

The results given in Tables I and II are typical for normal human samples.

Summary. By means of a titration method for estimation of bacterial growth it was found that growth is proportional to the quantity of nicotinamide present. Nicotinamide is more active than an

[8] Kohn, H. I., and Bernheim, F., *J. Clin. Invest.*, 1939, **18**, 585.

[9] Dorfman, A., Horwitt, M. K., Koser, S. A., and Saunders, F., *J. Biol. Chem.*, 1939, **128**, xx.

TABLE II.
Nicotinamide Content of Human Blood.

Sample	Dilution of filtrate	Method of sterilization	Value found, γ/cc whole blood	Hematocrit
A	.03	Filtered	.60	42.0
	.02		.54	
A	.03	Autoclaved	.80	42.0
	.02		.70	
B	.03	Filtered	.58	46.5
	.02		.55	
B	.03	Autoclaved	.88	46.5
	.02		.92	
C	.03	Filtered	.64	53.0
	.02		.72	
C	.03	Autoclaved	.91	53.0
	.02		.97	
D	.03	Filtered	.45	41.0
	.02		.52	
D	.03	Autoclaved	.83	41.0
	.02		.85	
E	.03	Filtered	.58	46.0
E	.03	Autoclaved	.75	46.0

equivalent amount of either pyridine-containing coenzyme. Hydrolysis increases the activity of the latter, indicating that the function of nicotinamide is not based entirely on synthesis to either of the known coenzymes. A method has been developed for determining nicotinamide and related substances in blood. The values obtained are higher if autoclaved blood is used.

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Rapidity of Passage of Chloride Ion from Blood into Gastric Juice of Stimulated Stomach.*

ALEXANDER BRUNSCHWIG AND ROBERT L. SCHMITZ.†

From the Department of Surgery and Division of Roentgenology, the Department of Medicine, The University of Chicago.

In order to study the passage of chloride from the blood into the acid gastric juice of the stimulated stomach, chloride ions were "tagged" by rendering them radioactive. Thus radioactivity detected in the juice would signify that such ions if previously injected into the blood had been brought through the gastric mucosa.

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† Research Assistant, International Cancer Research Foundation Grant.