

# THE FUNCTION OF NICOTINIC ACID IN BACTERIAL METABOLISM

I. J. KLIGLER AND N. GROSSOWICZ

*From the Department of Hygiene and Bacteriology, Hebrew University, Jerusalem*

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In our previous publications (Kligler and Grossowicz 1938, 1939) we showed that nicotinic acid plays an essential part in the carbohydrate metabolism of certain members of the colon-typhoid group of bacteria—particularly *Salmonella paratyphi A*, *Shigella dysenteriae* and some strains of the Flexner type of dysentery. Our results indicated that nicotinic acid was not a growth factor in the limited sense, because growth occurred in its absence, but that it played the part of a codehydrase making available to the organism a source of energy which it could not use in the absence of nicotinic acid.

The experiments reported in this paper were designed to test these findings and to account for the apparent contradiction between our results and those reported by Koser in his studies on *Shigella paradysenteriae*.

## EXPERIMENTAL

### *Methods*

The same medium was used as in the preceding study. The basic salt mixture was sterilized by filtration through a Seitz filter and a sterile solution of S-K peptone added to give a concentration of 0.3 per cent. This peptone proved useful for these studies because available tests showed that it was free of nicotinic acid, or at any rate that the amount was less than that demonstrable by bacteriologic methods. To this basic medium were added the substances to be tested. Since many of our experiments were made with lactate (which is fermented aerobically) small Erlenmeyer flasks were used instead of test tubes.

Since there might be some doubt as to whether the peptone used was really free of nicotinic acid some of the experiments were repeated in a medium of the same salt mixture plus the amino acids, Sodium Glutamate (mono), Asparagine and l-Cystine. *S. paratyphi A* grows well in this medium in the absence of nicotinic acid. Consequently it was possible to test the effect of this substance on growth and glycolysis.

In all other respects the procedure (preparation of inoculum, quantity, etc.) was the same as before.

The temperature of incubation was 30°C.

The growth density was determined by a photoelectric colorimeter constructed according to the design given by Evelyn, 1936. The cultures are placed in standard tubes and the amount of light passed through is measured against that passed through a control tube with sterile medium. The readings represent the per cent of light passed through to a photoelectric cell as indicated by the deflection of the needle on a sensitive galvanometer. The control tube reading is designated 100 and the others decrease with increasing turbidity. Comparable results can thus be obtained repeatedly with little expenditure of time and materials.

#### *Growth with and without nicotinic acid*

Our first problem was to ascertain the rôle of nicotinic acid in relation to growth in differently constituted media. Typical results are given in table 1. The strain used was *Salmonella paratyphi A*, the temperature 30°C. *Shigella dysenteriae* (Shiga) gave essentially the same results.

The data given in table 1 are typical of a large series of experiments. Because of their paradoxical nature we have retested the findings repeatedly with different concentrations of sugar and nicotinic acid, always with the same results. The noteworthy facts are: 1) Growth in non-carbohydrate media occurs in the absence of nicotinic acid; furthermore, in all experiments with the peptone medium there was poorer growth in the presence of nicotinic acid (compare final readings of 75 in the absence of nicotinic acid, with 78 in the presence of nicotinic acid). 2) Lactic acid is apparently used by this organism only slightly when there is no nicotinic acid present; the growth is poorer than

in the corresponding medium without lactate. 3) Glucose also is not used in the absence of nicotinic acid, but the curious point

TABLE 1  
*Growth density in different media with and without nicotinic acid*

MEDIUM	RESULTS AFTER . . . DAYS OF INCUBATION				
	2		3		6
	Photometer reading	Plate-count million per ml.	Photometer reading	Plate-count million per ml.	Photometer reading
1. Peptone media					
No carbohydrate, no nicotinic acid.....	83	338	78	282	75
No carbohydrate, + nicotinic acid.....	85	338	79	275	78
Sodium lactate 0.1 per cent, no nicotinic acid.....	87	200	86	208	85
Sodium lactate 0.1 per cent, + nicotinic acid.....	81	325	76	710	43
Glucose 0.1 per cent, no nicotinic acid.....	94	104	94	102	94
Glucose, 0.1 per cent, + nicotinic acid.....	59	1,160	57	1,210	42
Control (sterile).....	100		100		100
2. Synthetic media*					
No carbohydrate, no nicotinic acid.....	94	122	89	191	76
No carbohydrate, + nicotinic acid.....	91		85	328	66
Sodium lactate 0.1 per cent, no nicotinic acid.....	98	17	97	21.8	96
Sodium lactate 0.1 per cent, + nicotinic acid.....	74	145	60	765	48
Glucose 0.1 per cent, no nicotinic acid.....	100	3.9	100	4.6	100
Glucose 0.1 per cent, + nicotinic acid.....	60	670	58	1,370	46
Control (sterile).....	100		100		100

\* The quantities of amino acids used in our synthetic medium are: Asparagine 0.05 per cent, sodium glutamate 0.05 per cent, l-cystine 0.01 per cent.

is that the very presence of glucose prevents growth almost altogether unless nicotinic acid be present.

It seems, therefore, that nicotinic acid is not essential for growth either in the peptone or in the synthetic medium, and moreover that in its presence in our peptone non-carbohydrate medium, growth is slightly poorer than in the same medium without nicotinic acid. Furthermore, in the absence of nicotinic acid, glucose markedly inhibits growth; this inhibition is more marked in the synthetic than in the peptone medium.

In order to eliminate the possibility that these were aberrant observations, we tested the effects of different quantities of nicotinic acid on growth in the same peptone media, one series

TABLE 2

*Growth and fermentation of S. paratyphi A in media with and without nicotinic acid*  
pH 7.4; incubation 7 days; temperature 30°C.

Control (sterile).....	100	Control (sterile).....	100
0.1 per cent Na-lactate		No lactate	
- nicotinic acid.....	84	- nicotinic acid.....	79
- nicotinic acid.....	86	- nicotinic acid.....	78
+ nicotinic 100 $\gamma$ /ml.....	46	+ nicotinic 100 $\gamma$ /ml.....	86
+ nicotinic 20 $\gamma$ /ml.....	48	+ nicotinic 20 $\gamma$ /ml.....	83
+ nicotinic 10 $\gamma$ /ml.....	47	+ nicotinic 10 $\gamma$ /ml.....	84
+ nicotinic 10 $\gamma$ /ml.....	50	+ nicotinic 10 $\gamma$ /ml.....	84
+ nicotinic 4 $\gamma$ /ml.....	50	+ nicotinic 4 $\gamma$ /ml.....	85
+ nicotinic 0.8 $\gamma$ /ml.....	51	+ nicotinic 0.8 $\gamma$ /ml.....	84
+ nicotinic 0.16 $\gamma$ /ml.....	49	+ nicotinic 0.16 $\gamma$ /ml.....	86
+ nicotinic 0.032 $\gamma$ /ml.....	50	+ nicotinic 0.032 $\gamma$ /ml.....	81
+ nicotinic 0.0064 $\gamma$ /ml.....	62	+ nicotinic 0.0064 $\gamma$ /ml.....	80
+ nicotinic 0.0015 $\gamma$ /ml.....	76	+ nicotinic 0.0015 $\gamma$ /ml.....	79
+ nicotinic 0.0003 $\gamma$ /ml.....	84	+ nicotinic 0.0003 $\gamma$ /ml.....	79

without lactate, the other with 0.1 per cent Na-lactate. The results are given in table 2. It is evident that in the absence of lactate growth is poorer in the nicotinic-acid-containing flasks than in the controls, and that this difference persists almost to the point where the concentration of nicotinic acid is adequate to stimulate the active utilization of the lactate: 0.16 $\gamma$ /ml. in the former and 0.032 $\gamma$ /ml. in the latter.

It should be noted also that the titration curve of the amount of nicotinic acid required for lactate utilization corresponds exactly with that given by Dorfman, Koser *et al.* (1939), for

*S. paradysenteriae* (Flexner) in their synthetic glucose-containing medium. It is obvious, therefore, that our S-K peptone does not contain nicotinic acid. The conclusion drawn by Koser (1939) and his coworkers that nicotinic acid is essential for growth appears to be due to the fact that they were working with glucose-

TABLE 3

*Growth of S. paratyphi A in various carbohydrate media with and without nicotinic acid*

S-K peptone used. pH 7.4; incubation time, 5 days; Temperature, 30°C.

WITHOUT NICOTINIC ACID		WITH NICOTINIC ACID	
Control (sterile).....	100	Control (sterile).....	100
No carbohydrates.....	85	No carbohydrates.....	90
No carbohydrates.....	84	No carbohydrates.....	89
Glucose 0.01%.....	97	Glucose 0.01%.....	77
Glucose 0.05%.....	97	Glucose 0.05%.....	63
Glucose 0.2%.....	97	Glucose 0.2%.....	65
Galactose 0.2%.....	98	Galactose 0.2%.....	66
Galactose 0.2%.....	98	Galactose 0.2%.....	—
Trehalose 0.1%.....	98	Trehalose 0.1%.....	62
Trehalose 0.1%.....	97	Trehalose 0.1%.....	60
Mannitol 0.2%.....	97	Mannitol 0.2%.....	73
Mannitol 0.2%.....	97	Mannitol 0.2%.....	73
Sorbitol 0.2%.....	98	Sorbitol 0.2%.....	70
Sorbitol 0.2%.....	97	Sorbitol 0.2%.....	69
Na-lactate 0.1%.....	89	Na-lactate 0.1%.....	55
Na-lactate 0.1%.....	90	Na-lactate 0.1%.....	56
Na-acetate 0.3%.....	83	Na-acetate 0.3%.....	61
Na-acetate 0.3%.....	80	Na-acetate 0.3%.....	58
Maltose 0.2%.....	88	Maltose 0.2%.....	70
Maltose 0.2%.....	90	Maltose 0.2%.....	70
Sucrose 0.2%.....	83	Sucrose 0.2%.....	89
Sucrose 0.2%.....	85	Sucrose 0.2%.....	90

containing media only, and glucose in the absence of nicotinic acid has an inhibiting effect on growth.

In the preceding experiments it was noted that in the synthetic as well as in our peptone (S-K) medium, *glucose*, in the absence of nicotinic acid, apparently inhibits growth so that *visible growth* is not evident. That this is not an isolated phenomenon is demonstrated by the data given in table 3. In this experiment

we tested the effect of a variety of sugars some of which are and others are not fermented by our test organism. It will be noted that all the sugars fermented by *S. paratyphi* A., with the exception of maltose, inhibit growth in media without nicotinic acid, whereas sucrose which is not fermented does not exert this inhibiting effect. The phenomenon seems, therefore, to be a general one, the explanation of which still remains to be ascertained.

It is evident, however, that nicotinic acid is not essential for growth; its rôle in bacterial metabolism appears to be to activate carbohydrate metabolism, and by making available an easy source of energy it greatly increases the amount of growth in media containing these sources of energy.

#### *Relation of nicotinic acid to fermentation*

The subsequent experiments were designed to study more closely the function of nicotinic acid in carbohydrate fermentation. Having established that nicotinic acid functions not as a growth-promoting substance *per se* but as an activator of fermentation, we set out to ascertain at which stage of the carbohydrate breakdown it is essential. In the preceding publication it was shown that *S. paratyphi* A., *Shigella dysenteriae* (Shiga), as well as some of the Flexner strains, failed to ferment glucose in the absence of nicotinic acid. As a continuation of these experiments we now turned our attention to lactic acid. The same medium was used except that Na-lactate (0.1 or 0.2 per cent) served as a source of energy in place of glucose. *S. paratyphi* A. was used chiefly and the temperature of incubation was 30°C.

In the absence of nicotinic acid, in media containing phenol red as indicator, there was moderately good growth, but there was no change in reaction. In the presence of nicotinic acid growth was abundant and the reaction of the medium became markedly alkaline.

Analyses of the cultures showed that in the first instance (absence of nicotinic acid) only about 40 per cent of the lactic acid was used, whereas in the second all of it disappeared. The

lactic acid was determined by the method of Friedemann and Graesser (1933). It was found further that in the absence of nicotinic acid the lactate which disappeared could be accounted for entirely by volatile acids (determined by the Friedemann (1938) method), 80 per cent of which was acetic and 15–17 per cent was precipitated by  $\text{HgO}$ . In the presence of nicotinic acid the lactate was converted almost quantitatively to  $\text{CO}_2$ ; the alkalization of the medium was due to the carbonates formed.

As was also to be expected, the growth in the lactate medium in the absence of nicotinic acid was considerably less than in its presence. In the latter case there was also a tendency to pellicle formation. The reaction was entirely aerobic; under anaerobic conditions the lactic acid was not broken down. The experiments were carried out at pH 7.4 and 7.1, respectively, the latter being more satisfactory, because of the more complete utilization of the lactic acid, before inhibiting alkalinity developed. However, at pH 7.1 more  $\text{CO}_2$  is lost, and less recovered in the analyses than required by the amount of lactate consumed.

Another interesting point is that the amount of lactic acid consumed in the media not containing nicotinic acid was the same whether 0.1 or 0.2 per cent of lactate was used. This finding corresponds with those obtained with glucose. The pH cannot be the limiting factor, because there was no change in the reaction of the medium. At first we were inclined to the assumption that the accumulated acetic and formic (?) acid inhibited growth, but as we shall see below the concentration of volatile acid present was not sufficient to inhibit growth. The answer to this question remains, therefore, obscure.

A typical number of analyses of the lactic acid consumed are given in table 4. No comment is necessary. The differences are marked; fluctuations can be accounted for by differences in rate of growth, in degree of aeration, etc.

The differences in  $\text{CO}_2$  production are shown in table 5. In the absence of nicotinic acid the cultures did not contain more  $\text{CO}_2$  than did the control tubes. In the presence of nicotinic acid the

TABLE 4

*The utilization of lactic acid by S. paratyphi A in the presence and absence of nicotinic acid*

CONCENTRATION OF LACTATE	pH	CONTROL, LACTIC ACID	LACTIC ACID REMAINED	LACTIC ACID CONSUMED
<i>per cent</i>		<i>mgm.</i>	<i>mgm.</i>	<i>mgm.</i>
C 0.1	6.8	10.0	4.8	5.2
C 0.1	6.8	10.0	3.9	6.1
C 0.1	6.8	10.0	4.8	5.2
C 0.2	6.8	18.5	11.3	7.3
C 0.2	6.8	18.5	12.9	5.5
C 0.2	6.8	18.5	12.6	5.9
N 0.1	6.8	10.0	0.3	9.7
N 0.1	6.8	10.0	0.2	9.8
N 0.2	6.8	18.5	0.8	17.7
N 0.2	6.8	18.5	0.5	18.0
C 0.1	7.4	9.8	5.7	4.1
C 0.1	7.1	9.8	3.7	6.1
C 0.1	7.4	9.8	4.9	4.9
C 0.2	7.4	16.1	11.3	4.7
C 0.2	7.4	16.1	11.8	4.3
N 0.2	7.4	16.1	4.3	11.7

N = nicotinic acid present, C = nicotinic acid absent.

TABLE 5

*Products of oxidation of lactic acid in presence and absence of nicotinic acid*

EX- PERI- MENT	MEDIUM	pH		LACTIC ACID	LACTIC ACID RE- MAINED	LACTIC ACID CONSUMED	ACETIC ACID PRO- DUCED	CO <sub>2</sub> PRO- DUCED	EX- PECTED CO <sub>2</sub>
		Start	Finish						
1	(-) Nicotinic acid	7.1	7.2-7.3	10.0	5.22	4.78	3.0†	0	
2	(+) Nicotinic acid	7.1	9.0*	10.0	0.0	All con- sumed	0.55	11.83	14.7
3	(+) Nicotinic acid	7.1	9.0	10.0	0.0	All con- sumed	0.0	9.59	14.7
4	(+) Nicotinic acid	7.1	9.0	10.0	0.0	All con- sumed	0.0	7.17	14.7

\* Positive to phenolphthalein.

† 4.78 mgm. lactic acid = 3.15 mgm. acetic acid; the analysis gave 3.0 mgm.

oxidation of the lactic acid was complete and in some experiments the amount of CO<sub>2</sub> recovered represented 90 per cent of the lactic acid consumed.



In the absence of nicotinic acid only volatile acids and no CO<sub>2</sub> are produced.

These results suggested two possibilities:

1. That lactic acid can be oxidized without the mediation of nicotinic acid, but the process is quickly stopped by the accumu-

TABLE 6

*Growth density of S. paratyphi A in media containing different concentrations of acetic and formic acid with and without nicotinic acid*  
Incubation 7 days; pH 7.1; temperature 30° C.

WITHOUT NICOTINIC ACID		WITH NICOTINIC ACID	
Control.....	100	Control.....	100
No carbohydrates.....	75	No carbohydrates.....	84
Na-formate 0.04%.....	83	Na-formate 0.04%.....	85
Na-formate 0.07%.....	84	Na-formate 0.07%.....	86
Na-formate 0.10%.....	83	Na-formate 0.10%.....	86
Na-formate 0.30%.....	85	Na-formate 0.30%.....	—
Na-formate 0.50%.....	91	Na-formate 0.50%.....	86
Na-acetate 0.01%.....	75	Na-acetate 0.01%.....	77
Na-acetate 0.02%.....	75	Na-acetate 0.02%.....	75
Na-acetate 0.04%.....	73	Na-acetate 0.04%.....	67
Na-acetate 0.07%.....	73	Na-acetate 0.07%.....	66
Na-acetate 0.10%.....	76	Na-acetate 0.10%.....	52
Na-acetate 0.20%.....	77	Na-acetate 0.20%.....	45
Na-acetate 0.30%.....	76	Na-acetate 0.30%.....	45
Na-acetate 0.50%.....	80	Na-acetate 0.50%.....	44
Na-formate 0.01%.....	75	Na-formate 0.01%.....	72
Na-acetate 0.04%.....	75	Na-acetate 0.04%.....	72
Na-formate 0.02%.....	79	Na-formate 0.02%.....	64
Na-acetate 0.08%.....	79	Na-acetate 0.08%.....	64
Na-formate 0.04%.....	76	Na-formate 0.04%.....	53
Na-acetate 0.16%.....	76	Na-acetate 0.16%.....	53
Na-lactate 0.10%.....	82	Na-lactate 0.10%.....	46

lation of volatile acids, which cannot be oxidized further in the absence of nicotinic acid.

2. That the type of oxidation of lactic acid in the absence of nicotinic acid is essentially different from that with the intermediation of nicotinic acid.

The results of the experiment given in table 6 show that formic acid inhibits growth to some extent even in concentrations of 0.04 per cent. However, since nicotinic acid does not affect

the oxidation of formic acid, it is not likely that formic acid is produced in the lactic acid oxidation. Formic acid was not detected in the oxidation of lactic acid to  $\text{CO}_2$ . The  $\text{HgO}$  precipitate in the culture not containing nicotinic acid may represent some impurity.

On the other hand, acetic acid inhibits growth only when the concentration reaches 0.5 per cent, a concentration never reached in the oxidation of lactic acid in the absence of nicotinic acid. It seems, therefore, that the limiting factor is related to the type of oxidation. This is also supported by the fact that the amount of lactic acid consumed is not related to its concentration in the medium.

This experiment also shows that acetic acid oxidation cannot proceed at all in the absence of nicotinic acid. In its presence the growth density increases with the increased concentration of acetic acid, the reaction of the medium becomes alkaline as in the case of lactic acid, and the final oxidation product is  $\text{CO}_2$ .

*Comparative effect of nicotinic acid on aerobic and anaerobic fermentation*

The results of a typical experiment on the comparative effect of nicotinic acid under aerobic and anaerobic conditions, respectively, are given in table 7. Under anaerobic conditions lactic acid is not attacked even in the presence of nicotinic acid, and there is practically no growth whether nicotinic acid is present or not. Glycolysis proceeds under these conditions only to the point of lactic acid formation.

Under aerobic conditions, as previously noted, the events are quite different. In the lactate-containing media there is growth in the absence of nicotinic acid, but the density is decidedly less than in its presence. In the former case only volatile acids are formed, while in the latter the oxidation of volatile acids proceeds to the formation of  $\text{CO}_2$ . In glucose-containing media there is imperceptible growth in the absence of nicotinic acid, but dense growth and active fermentation of glucose with  $\text{CO}_2$  formation, in its presence.

Similar results were obtained with *Shigella dysenteriae* (Shiga).

The strain in our laboratory was obtained from London and designated as the Parker strain. It is the one used in our previous experiments. The growth density data given below (table 8)

TABLE 7

*Growth density of S. paratyphi A in various media under aerobic and anaerobic conditions*

pH 7.1; incubation time, 6 days; temperature, 30° C.

ANAEROBIC MEDIA	GROWTH DENSITY	AEROBIC MEDIA	GROWTH DENSITY
Control.....	100	Control.....	100
Na-lactate 0.1%, (-) nicotinic acid.....	99	Na-lactate 0.1%, (-) nicotinic acid.....	70
Na-lactate 0.1%, (+) nicotinic acid.....	99	Na-lactate 0.1%, (-) nicotinic acid.....	72
		Na-lactate 0.1%, (+) nicotinic acid.....	45
		Na-lactate 0.1%, (+) nicotinic acid.....	42
Glucose 0.1%, (-) nicotinic acid.....	93	Glucose 0.1%, (-) nicotinic acid.....	95
Glucose 0.1%, (+) nicotinic acid.....	78	Glucose 0.1%, (-) nicotinic acid.....	94
		Glucose 0.1%, (+) nicotinic acid.....	48
		Glucose 0.1%, (+) nicotinic acid.....	46
Glucose consumed:		No carbohydrate, (-) nicotinic acid.....	75
(-) nicotinic acid: 1.5 mgm.		No carbohydrate, (-) nicotinic acid.....	74
(+) nicotinic acid: 9.0 mgm.		No carbohydrate, (+) nicotinic acid.....	83
Lactic acid consumed		No carbohydrate, (+) nicotinic acid.....	84
(-) nicotinic acid: 0.0 mgm.			
(+) nicotinic acid: 0.0 mgm.			

show that essentially the same processes are involved. In the carbohydrate-free medium perceptible growth occurred both in the presence and absence of nicotinic acid. In glucose-containing media no perceptible growth occurred in the absence of nicotinic

acid, and fermentation and growth were active in the presence of nicotinic acid. In lactate-containing media growth occurred in the absence of nicotinic acid, but was denser in the presence of nicotinic acid; in the former the reaction was not changed, in the latter there was noticeable alkalization.

TABLE 8

*Growth density of Shigella dysenteriae (Shiga) in various media with and without nicotinic acid*

pH 7.4; incubation temperature, 37° C.; time, 6 days

MEDIA	GROWTH DENSITY
Sterile control.....	100
No carbohydrate	
+ nicotinic acid.....	86
- nicotinic acid.....	90
Glucose	
+ nicotinic acid.....	72
+ nicotinic acid.....	70
- nicotinic acid.....	97
- nicotinic acid.....	96
Lactate 0.1%	
+ nicotinic acid.....	69
+ nicotinic acid.....	65
- nicotinic acid.....	88
- nicotinic acid.....	87

#### *The function of nicotinic acid in respiration*

It now remained to ascertain whether nicotinic acid acts as such or as a part of cozymase. It is accepted that dehydrogenation of lactic acid proceeds with the aid of cozymase (cozymase-linked dehydrogenases). Lwoff and Lwoff (1937), working with *Hermophilus parainfluenzae* and using the Thunberg technique showed that when these organisms were grown on media without the "V" factor their rate of respiration could be greatly increased by the addition of codehydrase, coenzyme I or II. Using a similar technique we carried out a series of experiments which indicated that nicotinic acid added to such a system stimulates respiration only after it is converted to another substance, probably cozymase. However, while these experiments were in

progress, Dorfman *et al.*, 1939, reported results which suggested that nicotinic acid or nicotinamide stimulated respiration directly in the same manner as cozymase. We, therefore, repeated our experiments, following closely the technique used by these authors.

The procedure used was briefly as follows:

Cultures of *S. paratyphi A.* were grown in four 250 ml. flasks, each containing 100 ml. of our basic medium plus 0.1 per cent Na-lactate. To two of the flasks were added 0.5 $\gamma$  nicotinic acid (0.005 $\gamma$  per ml.). The flasks were incubated about 60 hours at 30°C. At the end of the period the organisms were centrifuged, washed in slightly buffered saline and resuspended in M/15 phosphate buffer of pH 7.4. The density of the suspensions was standardized to give a reading of 47 in the photometer cell; this concentration was used because it was the maximum reading obtained in our cultures.

The respiration experiments were conducted either in Thunberg tubes, or in ordinary test tubes covered with 5 ml., boiling vaseline, cooled quickly and placed in a 37°C. water bath. The two methods gave identical results. The following system was used:

	ml.
M/15 phosphate buffer pH 7.4.....	1.0
1/5000 Methylene blue.....	1.0
Bacterial suspension.....	1.5
M/20 Na-lactate or M/20 glucose.....	1.0
Distilled water.....	0.5
Total <sup>1</sup> .....	5.0

Controls contained the same materials with the exception of the donator. Nicotinic acid and cozymase<sup>2</sup> were added as indicated in each experiment.

<sup>1</sup> In Thunberg tubes we used half the quantities given above.

<sup>2</sup> The cozymase was prepared from bakers' yeast according to Euler and Karlsson. It was only partially purified: extracted with distilled water, proteins precipitated with lead acetate and the filtrate treated with NaOH to a light blue to thymolphthalein; the precipitate formed was taken up in  $\frac{1}{10}$  the original volume of distilled water and lead removed with H<sub>2</sub>S. The filtrate was adequately pure for our purpose since filtrate controls were used as a check in each experiment.

The results of a typical experiment are shown in table 9. Comparable results were obtained in several experiments made at different times.

The following points may be deduced from the respiration experiments:

1. When cozymase was added to the system the respiration rate was the same whether the bacterial suspension was prepared from cultures grown in a medium with or without nicotinic acid. Be-

TABLE 9  
*The effect of nicotinic acid on respiration of resting bacteria*

AMOUNT OF NICOTINIC ACID ADDED $\gamma$ /ML. TO RESPIRATION SYSTEM	CULTURE SUSPENSIONS USED IN THIS TEST WERE GROWN IN A LACTATE MEDIUM IN PRESENCE OF 0.005 $\gamma$ /ML. NICOTINIC ACID		CULTURE SUSPENSIONS USED IN THIS TEST WERE GROWN IN THE SAME LACTATE MEDIUM WITHOUT NICOTINIC ACID	
	M/20 lactate as donator Reduction time of M.B. 1/5000	M/20 glucose as donator Reduction time of M.B. 1/5000	M/20 lactate as donator Reduction time of M.B. 1/5000	M/20 glucose as donator Reduction time of M.B. 1/5000
	<i>min.</i>	<i>min.</i>	<i>min.</i>	<i>min.</i>
100	20	14	35	22
20	24	17	40	26
4	28	25	53	41
0.8	33	26	92	71
0.0	61	46	No reduction	No reduction
Cozymase 0.2 ml.	14	10	16	13
Controls:				
1. no donator	No reduction	No reduction	No reduction	No reduction
2. no donator + 20 $\gamma$ nicotinic acid	No reduction	No reduction	No reduction	No reduction
3. no donator + 0.2ml. cozymase	23	23	28	28

cause the cozymase preparation was impure reduction of methylene blue also occurred in the absence of the donator, but the time interval was about twice as long.

2. When nicotinic acid was added to the system in place of cozymase the reduction time was significantly greater in the case of suspensions grown in media without than in those grown in media with nicotinic acid. In other words, in the former case a longer "incubation" period was required before the nicotinic

acid reached a state where it could influence the respiration. Glucose is a better donator than lactic acid, but the results were basically similar.

3. Moreover in the respiration systems to which no nicotinic acid was added, suspensions of bacteria grown in the presence of minimal amounts (0.005 $\gamma$ ) of nicotinic acid activated reduction, although it was slow; while suspensions of bacteria grown in

TABLE 10

*Effect of bacterial extracts (autolysate) on the reduction rate in a dehydrogenase system. (Na-lactate as donator)*

FERMENT USED	SUBSTANCE ADDED TO THE RESPIRATION SYSTEM	REDUCTION TIME
		<i>min.</i>
Lacticodehydrase:		
Before dialysis.....	Cozymase 0.5 ml.	90
Before dialysis.....	Cozymase 0.5 ml.	95
Before dialysis.....	Nicotinic acid 100 $\gamma$ /ml.	180
Before dialysis.....	Nicotinic acid 20 $\gamma$ /ml.	150
Before dialysis.....	Nothing added	150
Before dialysis.....	Control (without lactate)	No reduction
After dialysis.....	Cozymase 0.5 ml.	300
After dialysis.....	Cozymase 0.5 ml.	320
After dialysis.....	Nothing added	No reduction
After dialysis.....	Nothing added	No reduction
After dialysis.....	Nicotinic acid 100 $\gamma$ /ml.	No reduction
After dialysis.....	Nicotinic acid 20 $\gamma$ /ml.	No reduction
After dialysis.....	Cozymase 0.5 ml.	No reduction
After dialysis.....	Control (without lactate)	No reduction

media without nicotinic acid failed to cause reduction of methylene blue even after 24 hours.

It appears then that there is a lag period before nicotinic acid can activate the dehydrogenation system. It is also apparent that bacteria grown in media containing even such minimal amounts of nicotinic acid as 0.5 $\gamma$  per 100 ml. carry with them the activator so that reduction of methylene blue in the respiration system occurs even if no cozymase or nicotinic acid is added. The conclusion that nicotinic acid as such activates respiration

is invalid if the organisms used in the suspension were grown in a medium containing even minimal amounts (0.005 $\gamma$ /ml.) of nicotinic acid.

A further proof that the nicotinic acid is converted into some other substance (codehydrase) before it becomes active was furnished by the following experiment. Cultures were grown on standard nutrient agar made with Difco peptone to which 0.5 per cent Na-lactate was added. The growth was washed off with saline, centrifuged, washed twice and then suspended in M/2 phosphate buffer. The suspension was allowed to autolyze 5 days at 37°C., centrifuged and a clear supernatant extract obtained. This fluid should, according to Stephenson (1928), contain specific lacticodehydrase.

The extract was divided into two parts, one of which was dialysed for about 14 hours, and the dialysed and undialysed fractions tested in the respiration system described above, the extract taking the place of the bacterial suspension. The results are shown in table 10.

It is evident that addition of nicotinic acid to the undialysed fraction does not affect the reduction time, whereas cozymase does. In the dialysed fraction addition of cozymase activates reduction even though the time is prolonged, whereas addition of nicotinic acid has no effect at all. It appears, therefore, that the conversion of nicotinic acid to an activator (codehydrase) occurs in the living cell.

#### DISCUSSION

In the present investigation we set out to ascertain the rôle played by nicotinic acid in bacterial growth and metabolism, or more correctly in the metabolism of some of the members of the colon-typhoid group of bacteria. It appeared to us that the elucidation of the function of this vitamin in the physiology of the bacterial cell might suggest also its rôle in animal physiology. At the outset the question was somewhat confused by the claim that nicotinic acid was a specific growth factor without which no growth occurs. However, our results indicated that, at least insofar as *paratyphoid A* was concerned, growth was possible in



nutrient media made up of amino acids or of a peptone shown to be devoid of nicotinic acid, and that the latter substance played an essential rôle only in the presence of fermentable carbohydrates. In this respect our findings differed from those of Koser and his coworkers.

The data reported in this paper offer an explanation for the disparity of the findings. Our experiments demonstrate four essential facts. 1) that *Salmonella paratyphi A* can grow fairly well in a medium composed of amino acids or of a nicotinic-acid-free peptone, whether or not nicotinic acid is added; 2) that the addition of fermentable carbohydrates to such a medium free of nicotinic acid inhibits growth; 3) that nicotinic acid becomes effective as a stimulant only when a fermentable carbohydrate or a utilizable organic acid is present in the medium; 4) that the nicotinic acid is apparently first converted by the cell into some active substance (codehydrase) before it becomes active.

The inhibitive effect of fermentable carbohydrates (in the absence of nicotinic acid) on growth appeared at first anomalous. No such observation had previously been reported. However, repeated tests yielded the same results. It was this peculiar effect which accounted for the differences between Koser's findings and his interpretation and our own.

A probable explanation of this phenomenon is suggested by the results recently reported by Rosenthal *et al.* (1940). These authors showed that in certain tissues possessing the power of aerobic glycolysis, glucose suppresses the respiration in a manner similar to that observed by Crabtree with tumor tissue. In the case of cartilage which has a low respiration and hence a higher aerobic glycolysis, glucose retards oxygen consumption by 63 per cent; a similar effect was obtained with mannose but not with fructose which is not glycolysed by cartilage. These authors state: "apparently the addition of glucose suppresses cellular processes which result in the formation of pyruvic and succinic acids." Dickens and Greville (1933) showed that the oxidation of glucose rather than its glycolysis was responsible for the retardation of protein catabolism observed by Warburg. It appears that similar circumstances exist in the case of bacteria

grown in carbohydrate media without nicotinic acid. In the absence of this substance these organisms are deprived of their anaerobic glycolytic power; only a low rate of aerobic glucose oxidation occurs which presumably retards protein catabolism. If we assume that the rate of glucose oxidation by the cell is slower than that of the amino acids then we have an explanation of the retarding effect of glucose on growth (in the absence of nicotinic acid). In other words the phenomenon observed by us is the "Crabtree effect" among bacteria limited to aerobic glycolysis, because of the absence of nicotinic acid.

The inhibitive effect produced by glucose can be duplicated with any carbohydrates fermented by this organism, but not with sucrose which is not fermented. This effect is less marked in the case of lactic acid and of maltose which are apparently more readily oxidised aerobically than glucose.

It appears, therefore, that there is no contradiction between Koser's experimental findings and our own; the differences lie in the experimental procedure and in the interpretation. Our controlled results in different media make it clear that nicotinic acid plays an essential rôle in the fermentation of carbohydrates.

It is also of interest that nicotinic acid is required not only for the fermentation of carbohydrates but also for the oxidation of the acids resulting from their decomposition. In the absence of nicotinic acid lactic acid can be only partly oxidised to acetic acid and acetic acid not at all. Nicotinic acid is essential for the oxidation of acetic acid to  $\text{CO}_2$ . In general it appears that this accessory substance is necessary for the proper utilization of the carbon sources of energy. Without it, with the organisms studied, there is no fermentation.

Finally we tried to ascertain whether nicotinic acid functions as such or as a part of cozymase. It appears to us that our dehydrogenation experiments give an unequivocal answer to this question. Organisms grown in media not containing nicotinic acid cannot act as activators in the dehydrogenation of either glucose or lactic acid; whereas those grown in media containing minimal amounts of nicotinic acid and glucose or lactic acid and treated in the identical manner do act as activators. Nicotinic

acid added to the respiration system acts 2 to 3 times as slowly in the former case as in the latter. The reasonable explanation of this difference in behavior is that nicotinic acid does not act as such but is first converted by the cell into cozymase or codehydrase.

The mechanism of action of nicotinic acid appears, therefore, clear. It is converted by the cell into cozymase (codehydrase) which plays an essential part in the utilization of fermentable carbon compounds. The ready source of energy thus made available provides the possibility for increased growth activity. It may be assumed that its function in the animal cell does not differ essentially from that in the bacterial cell.

#### CONCLUSIONS

Using *Salmonella paratyphi A* chiefly as the test organism, it was found that:

1. Nicotinic acid added in varying amounts to a carbohydrate-free synthetic or peptone medium (free of nicotinic acid) does not appreciably affect growth. In some media somewhat better growth is obtained without nicotinic acid, in others, the reverse is the case.

2. In the absence of nicotinic acid, glucose (and other fermentable carbohydrates) added to such a medium inhibits or may even prevent growth. Lactate does not exert this effect or only to a lesser degree.

3. Nicotinic acid is not a specific growth-promoting substance. It plays an essential rôle in the fermentation of carbohydrates. It is also essential for the complete utilization of lactic acid and more particularly for the oxidation of acetic acid. It does not seem to play a part in the oxidation of formic acid by this organism.

4. Dehydrogenation experiments indicate that nicotinic acid does not act as such but must first be converted to a cozymase-like substance (codehydrase). Such conversion occurs only in the living cell.

## REFERENCES

- CRAFTREE, H. G. 1929 Observations on the carbohydrate metabolism of tumours. *Biochem. J.*, **23**, 536-545.
- DICKENS, F., AND GREVILLE, G. 1933 Metabolism of normal and tumour tissue. *Biochem. J.*, **27**, 1123-1140.
- DORFMAN, A., KOSER, S. A., REAMES, H. R., SWINGLE, K. F., AND SAUNDERS, F. 1939 Nicotinamide and related compounds as essential growth substance for dysentery bacilli. *J. Infectious Diseases*, **65**, 163-182.
- DORFMAN, A., KOSER, S. A., SAUNDERS, F. 1939 Effect of nicotinamide on respiration of dysentery bacilli. *Science*, **90**, 544-545.
- EVELYN, K. A. 1936 A stabilized photoelectric colorimeter with light filters. *J. Biol. Chem.*, **115**, 63-75.
- FRIEDEMANN, T. E., AND GRAESER, J. B. 1933 The determination of lactic acid. *J. Biol. Chem.*, **100**, 294-308.
- FRIEDEMANN, T. E. 1938 The identification and quantitative determination of volatile alcohols and acids. *J. Biol. Chem.*, **123**, 161-184.
- KLIGLER, I. J., AND GROSSOWICZ, N. 1938 Nicotinic acid and the fermentation of dextrose by the colon-typhoid group of bacteria. *Nature*, **142**, 76-77.
- KLIGLER, I. J., AND GROSSOWICZ, N. 1939 The influence of nicotinic acid on glucose fermentation by members of the colon-typhoid group of bacteria. *J. Bact.*, **38**, 309-320.
- KOSER, S. A., DORFMAN, A., AND SAUNDERS, F. 1938 Nicotinic acid as an essential growth substance for dysentery bacilli. *Proc. Soc. Exptl. Biol. Med.*, **38**, 311-313.
- KOSER, S. A., FINKLE, R. D., DORFMAN, A., GORDON, M. V., AND SAUNDERS, F. 1938 A comparative study of the growth promoting properties of various substances. *J. Infectious Diseases*, **62**, 209-218.
- LWOFF, A., AND LWOFF, M. 1937 Studies on codehydrogenases. *Nature of factor "V"*. *Proc. Roy. Soc. (London)*, **B, 122**, 352-373.
- ROSENTHAL, O., BOWIE, M. A., AND WAGONER, G. 1940 On the interdependence of respiration and glycolysis. *Science*, **92**, 382-383.
- STEPHENSON, M. 1928 A cell free enzyme preparation obtained from bacteria. *Biochem. J.*, **22**, 605-614.