THE INFLUENCE OF NICOTINIC ACID ON GLUCOSE FERMENTATION BY MEMBERS OF THE COLON-TYPHOID GROUP OF BACTERIA[1]

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Knowledge concerning the enzymic systems of various pathogenic bacteria and their dependence on specific vitamins or accessory substances is still rather vague. The work of Davis (1917), Thjötta and Avery (1921), and Fildes (1921) has demonstrated that *Hemophilus influenzae* requires two substances for its growth: x, a heat-stable substance, probably hematin, and v, a heat-labile substance. Kligler (1919) showed, in an extended study on the growth requirements of certain pathogenic bacteria (*Corynebacterium diphtheriae*, meningococcus, etc.), that minute quantities of tissue extractives, or extracts of nasal mucous secretion are either essential for growth or have a stimulating effect. More recently Knight (1937) has shown that nicotinic acid was essential for the growth of *Staphylococcus aureus*, and

[1] Note: The experiments reported in this paper were carried out with the Schering-Kahlbaum peptone für bacteriologische Zwecke. Since the paper was submitted for publication, we changed over to Difco peptone and failed to duplicate the results. A comparative test with three peptones showed that work on the effect of nicotinic acid can only be carried out satisfactorily with synthetic media or with peptone free from nicotinic acid. The fermentation of glucose in media made with the different peptones is summarized below. The media contained 0.3 per cent peptone, 0.1 per cent glucose, salt solution as given in the body of the paper, and 10γ nicotinic acid per ml.:

	SCHER.-KAHL		RIEDEL		DIFCO	
	+N	−N	+N	−N	+N	−N
Shigella dysenteriae.....	+	−	+	+	+	+
Salmonella paratyphi A....	+	−	+	+	+	+

Mueller (1937) has reported that *C. diphtheriae* can be cultivated on synthetic media if nicotinic acid and β alanin are added. Since nicotinic acid forms a part of the fermentation enzyme of Warburg and functions as a cozymase, we have undertaken a study of the relation of this substance, if any, to the fermentative capacities of the various species of the colon-typhoid-dysentery group of bacteria. A preliminary note dealing principally with *Shigella dysenteriae* (Shiga), has already been published (Kligler and Grosowitz, 1938). The object of this paper is to report the results of experiments with representative species of this group of fermenting bacteria.

EXPERIMENTAL

Methods

In order to exclude, in so far as possible, unknown substances and at the same time avoid expensive and complicated synthetic substrates, the experiments were conducted with a semi-synthetic medium. The basis of this medium was peptone, which provided a mixture of the various amino-acids required for growth; all the other ingredients were chemically pure substances. The basic solutions used throughout these experiments consisted of the following:

A. Salt mixture:

	per cent
NaCL	0.5
$Na_2HPO_4 \cdot 2H_2O$	0.25
KH_2PO_4	0.035
$MgCl_2$	0.03
Fe and Mn	trace

These salts were dissolved in distilled water and filtered through a Seitz filter.

B. A stock solution of peptone dissolved in distilled water and autoclaved.

C. The glucose and vitamin solutions respectively were dissolved in distilled water and sterilized by filtration through Seitz filters.

The liquid media were prepared by adding desired amounts of

solutions B and C to the stock salt solution. Solid media were prepared by adding the requisite amount of a stock solution of agar in distilled water, to give a concentration of two per cent. The agar-agar used was first thoroughly washed with distilled water, dried and then the stock solution made up in distilled water.

Fermentation in solid media

An orientation experiment was made by growing the various strains on a solid medium consisting of the stock salt solution,

TABLE 1

Growth and glucose fermentation in the experimental solid medium with and without nicotinic acid

STRAIN	GROWTH AND FERMENTATION		
	Without nicotinic acid	With nicotinic acid	Russell double sugar
Eberthella typhosa H 441	+	+	+
Salmonella paratyphi A	0	+	+G
Salmonella paratyphi B	+G	+G	+G
Escherichia coli	+G	+G	+G
Shigella dysenteriae Shiga (Parker strain)	0	+	+
Shigella dysenteriae Flexner	−	+	+
Shigella dysenteriae Y	−	+	+

Note: 0 = no growth; − = poor growth, no acid; + = growth & acid formation; G = gas.

0.3 per cent peptone, 0.2 per cent glucose, 2 per cent agar and andrade indicator. One set of tubes contained nicotinic acid, the other did not. The usual stab and slant inoculations were made as in Russell double sugar media. Ordinary nutrient double sugar tubes were inoculated for comparison. The results are summarized in table 1. It is apparent from the data that there are marked differences in the behavior of the different species of bacteria. In the medium without nicotinic acid the dysentery bacilli as a group either fail to grow or grow poorly without evidence of fermentation of the glucose. The same is true of paratyphoid A. The other species tested grew well and

were able to ferment glucose without the aid of nicotinic acid. It is of interest also to note that paratyphoid A produced acid but no gas in the synthetic medium containing nicotinic acid. These points will be dealt with in detail below.

It would seem that this relatively simple semi-synthetic medium with glucose and andrade indicator may serve to differentiate paratyphoid A from B and *S. dysenteriae* from *E. typhi.*

In order to ascertain whether the paratyphoid A and dysentery strains required vitamins or growth-stimulating substances other than nicotinic acid, these strains were grown on the stock

TABLE 2

Growth and glucose fermentation in media containing various vitamins with and without nicotinic acid

	SHIGA	FLEXNER	PARATYPHI A
Without nicotinic acid:			
Control	0	−	0
1γ/ ml. B_1	0	−	0
10γ/ml. B_2	0	−	0
10γ/ml. β alanin	0	−	0
10γ/ml. β alanin + B_2	0	−	0
200γ/ml. ascorbic acid	−	−	−
With nicotinic acid:			
10γ/ml. Nicotinic acid	+	+	+
N + B_1	+	+	+
N + β alanin	+	+	+
N + ascorbic acid	+	+	+
N + β alanin + B_2	+	+	+

medium described above, to which various substances were added. The results are summarized in table 2. None of the accessory substances added, with the exception of nicotinic acid, had any effect on the growth or fermentation of the bacteria used. As in the preceding experiment the dysentery strains grew in all media not containing nicotinic acid but did not produce acid. On the other hand, in the corresponding media containing nicotinic acid, all strains grew much more abundantly with the production of acid along the stab inoculation. It should be noted that in this series of media also the paratyphoid A strain failed to produce gas.

A clearer picture of the influence of nicotinic acid on those bacteria which failed to give evidence of fermentation in the solid glucose medium devoid of this substance, was obtained by noting the acid production and sugar utilization in liquid media. The medium was prepared in the manner described above. To avoid carrying over traces of accessory substances with the bacteria, small inocula were used (about 100 cells) of a broth culture diluted in saline. Where for comparison larger inocula (1 million cells) were used, the broth cultures were washed twice in saline and saline suspensions used for inoculation. All experiments were repeated at least once. Since there was practically no deviation in the results, only typical experiments are given.

The effect of various concentrations of peptone on the amount of sugar utilized

This experiment is illustrative. The media were the same in every other respect, except that they contained varying concentrations of peptone. The same inoculum was used. The glucose was determined by the Lehmann-Maquenne method.

The results are summarized in table 3. It will be noted that, to a limited extent, the concentration of peptone influenced the degree of sugar utilization. This is presumably due to the effect of peptone on the rate of growth, since more prolonged incubation decreased the differences. However, the data, whether after twenty-four or forty-eight hours incubation, show clearly that in the absence of nicotinic acid there is a small, though constant destruction of glucose (11 per cent) in all tubes, whereas in the presence of nicotinic acid over 90 per cent of the glucose is utilized in all tubes containing 0.2 per cent peptone or over. Longer incubation does not affect these results.

It is difficult to account for the small but consistent destruction of glucose in the absence of nicotinic acid. As already stated, the fact that even this degree of utilization is influenced by the concentration of peptone and the incubation time, would indicate that the loss is due to cell activity. It must be assumed, however, that this glycolysis differs in character from that taking place in the presence of nicotinic acid.

Experiments to determine the effect of nicotinic acid on cell activity as shown by the duration of the lag period did not always give consistent results. In general there were indications of a small decrease in the duration of the lag in media containing nicotinic acid, but the differences were not sufficiently marked to suggest that this substance was essential for stimulating cell metabolism other than as a cozymase in glucose fermentation.

TABLE 3

Influence of nicotinic acid on the utilization of glucose by S. dysenteriae (Shiga), in media containing varying concentrations of peptone

INCUBATION TIME AT 37°C.	CONCENTRATION OF PEPTONE	GLUCOSE REMAINING IN 9 ML. OF MEDIA:			
		With nicotinic acid		Without nicotinic acid	
hours	*per cent*	*mgm.*	*per cent*	*mgm.*	*per cent*
24	0.1	4.96	60.0	8.24	98.0
	0.2	1.82	22.0	7.50	90.0
	0.3	0.65	8.0	7.25	89.0
	0.4	0.56	7.0	7.18	89.0
	0.5	0.80	8.0	7.19	89.0
48	0.1	3.99	47.0	7.24	89.0
	0.2	0.76	9.0	7.24	89.0
	0.3	0.62	7.0	7.25	89.0

The results of a typical count are given below. The media contained 0.3 per cent peptone. To one set β alanin was also added.

MEDIUM	WITHOUT NICOTINIC ACID; COUNT PER ML. AFTER HOURS					WITH NICOTINIC ACID (10γ/ML.) COUNT PER ML. AFTER HOURS:				
	0	2	4	6	8	0	2	4	6	8
Stock exper. medium.....	50	75	87	590	4,000	64	111	242	1,690	8,600
Stock + 10γ/ml. β alanin.....		43	127	720	4,500		72	247	1,650	8,900

Influence of nicotinic acid on the utilization of glucose by various species of dysentery bacilli (other than Shiga)

In the preliminary tests in the solid medium the Flexner strain used behaved in the same manner as did the Shiga strain. It was of interest, however, to note whether all types of dysentery

bacteria require nicotinic acid. The procedure was the same as in the preceding experiments. The medium contained 0.3 per cent peptone and 0.1 per cent glucose. The cultures were incubated for 48 hours at 37°C. and the glucose content determined. The results are shown in table 4. It will be noted that all of the type cultures used with the exception of two strains of Flexner failed to ferment glucose in the absence of nicotinic acid. The two strains which did not require nicotinic acid, fermented mannitol and maltose and were agglutinated to the full titre by a specific Flexner serum. The tests were repeated in liquid and solid media with the same results. It would seem, therefore,

TABLE 4

Influence of nicotinic acid on the fermentation of glucose by various dysentery bacteria (incubation 48 hours at 37°C.)

Strain	Glucose remaining in 9 ml. of media:				Growth in solid media	
	With nicotinic acid		Without nicotinic acid		With N acid	Without N acid
	mgm.	*per cent*	*mgm.*	*per cent*		
Flexner	0.12	1.0	7.88	93.0	+	−
Flexner x	0.30	3.0	7.75	91.0	+	−
Flexner z	0.20	2.0	0.35	3.0	+	+
Flexner w	0.0	0.0	0.2	2.0	+	+
Strong	0.5	7.0	6.84	81.0	+	−
Hiss-Y	0.64	8.0	7.44	89.0	+	−
Schmitz	0.70	8.0	7.80	93.0	+	0

that the Flexner strains vary in their need of nicotinic acid and represent in this respect an intermediate or transitional stage in the loss of ability to produce the cozymase. Those strains which corresponded with the Shiga type gave the same results: in the absence of nicotinic acid a small, limited, portion of the glucose disappeared but the process always stopped at the same point.

Influence of nicotinic acid on glucose fermentation by S. paratyphi A.

The preliminary tests on the solid semi-synthetic medium indicated that paratyphoid A differs from the other typhoid-paratyphoid bacteria in its inability to ferment glucose in the absence of nicotinic acid. The following are the results of a typical

analysis in the liquid medium containing 0.3 per cent peptone and 0.1 per cent glucose. The period of incubation was forty-eight hours at 37°C.

	WITHOUT NICOTINIC ACID, GLUCOSE REMAINING		WITH NICOTINIC ACID, GLUCOSE REMAINING	
	mgm.	*per cent*	*mgm.*	*per cent*
E. typhi 441	1.14	13.0	0.35	4.0
S. paratyphi A	6.64	79.0	0.64	7.0

An interesting problem was raised by the fact that in the medium used in these experiments, even when nicotinic acid was added and the sugar was practically completely utilized, no gas was formed. At first it was assumed that another substance was lacking in our medium which was present in nutrient broth—a carboxylase, which the organism could not synthesize. However, a variety of experiments showed that the controlling factor was the temperature of incubation. When the culture was grown in fermentation tubes in nutrient glucose broth and duplicate tubes incubated at 30 and 37°C. respectively, only a small amount of gas was produced at 37°C. and at least four to five times as much at 30°C. In the synthetic medium the differences were even more marked; as a rule no gas appeared at 37°C., while there was an appreciable amount at 30°C.

Table 5 summarizes the results of several experiments. The medium contained 0.3 per cent peptone and 0.2 per cent glucose. The incubation time was 48 hours. The inocula, etc. in the tubes incubated at the different temperatures were always identical.

The addition of β-alanin, d-alanin and B_1, singly and in combination, did not alter the results.

In order to rule out the possibility that these results were only apparent, due to a greater solubility of the CO_2 at 37°C., we determined the glucose consumed and the lactic acid produced at the two temperatures. The analyses were as a rule made in media containing 0.3 per cent peptone and 0.2 per cent glucose. For comparison, analyses were also made in media containing only 0.1 per cent glucose. The average results of a large series of experiments are summarized in table 6.

It will be noted that while 0.1 per cent glucose (1 mgm./ml.) is completely used up at both temperatures, the amount of lactic acid produced at 37°C., is one and a half times that at 30°C. When 0.2 per cent glucose is used, more sugar is consumed at 30°C. than at 37°C., with more acid produced at the latter temperature; in this case also, the amount of lactic acid produced at 37°C. is about 50 per cent greater than at 30°C.

As in the previous experiments, a certain amount of glucose is used up in the nicotinic-acid-free medium. The amount used is

TABLE 5

Gas production in the nicotinic acid medium in fermentation tubes incubated at 30 and 37°C. respectively

TEMP. OF INCUBATION	EXPER. MEDIUM	WITH NICOTINIC ACID	WITHOUT NICOTINIC ACID
°C.			
30	1	Growth good, 1.1 ml. gas	Growth poor, no acid, no gas
	2	2.0 ml. gas	Same as one
	3	0.9 ml. gas	Same as one
	4	0.6 ml. gas	
	Broth	3.0 ml. gas	3.0 ml. gas
	Broth	3.1 ml. gas	3.1 ml. gas
	Broth	2.1 ml. gas	
37	1	Growth good, acid, no gas	Growth good, no acid, no gas
	2	Growth good, 0.3 ml. gas	Growth good, no acid, no gas
	3	Growth good, acid, no gas	Growth good, no acid, no gas
	4	Growth good, acid, no gas	Growth good, no acid, no gas
	Broth	0.5 ml. gas	0.5 ml. gas
	Broth	0.8 ml. gas	0.8 ml. gas
	Broth	0.5 ml. gas	

small and is the same at both temperatures. It may well be that in addition to glucose fermentation, the organisms tested are also capable of a limited direct attack on the glucose molecule. This, however, is a matter for future investigation.

The only other organism of this group which showed this peculiar effect of temperature was Paratyphoid C. This organism does not require nicotinic acid. On the other hand, it uses up more sugar and produces more gas at 30°C. than it does at 37°C. No differences, however, were found in the quantities of

lactic acid produced. The average results of a series of experiments in our semi-synthetic medium containing 0.3 per cent glucose are summarized below. There were, of course, differ-

TABLE 6

Glucose consumed and lactic acid produced by S. paratyphi A. at 30 and 37°C. respectively; incubation period 48 hours

CONCENTRATION OF SUGAR	30°C.				37°C.			
	Glucose consumed; mgm./10 ml.		Lactic acid prod.; mgm./10 ml.		Glucose consumed; mgm./10 ml.		Lactic acid prod.; mgm./10 ml.	
	Nic.+	Nic.−	Nic.+	Nic.−	Nic.+	Nic.−	Nic.+	Nic.−
per cent								
0.1	Complete	0.65	1.23		Complete	1.21	2.00	
0.2	15.50	1.25	3.21	0.24	13.40	1.32	4.54	0.26

ences between individual experiments, but the trend was always the same and the averages represent the characteristic findings:

TEMPERATURE	SUGAR CONSUMED; MGM./10 ML.	LACTIC ACID PRODUCED; MGM./10	GAS PRODUCED; ML.
°C.			
30	21.27	3.43	2.3
37	17.82	3.37	1.6

It will be noted that the temperature effect is not as marked as in the case of Paratyphoid A; nevertheless, there is a distinct difference in the rate of fermentation at the two temperatures.

These results indicate that there is a new factor influencing the fermentation of glucose by these paratyphoid organisms which has not hitherto been taken into account. This effect is most marked in Paratyphoid A. It is difficult to explain how the temperature inhibits the further breakdown of the intermediate products of glucose fermentation, but the results are quite clear and consistent. At 37°C. the fermentation proceeds to the acid stage and the glycolysis continues until the accumulated acid inhibits further growth. At 30°C., on the other hand, the acid products are more readily broken down to gas, growth proceeds further and more sugar is utilized than at 37°C. This accounts

also for the fact that in the medium used growth was always better at 30°C. than at 37°C.

DISCUSSION

The results presented in this paper indicate that there are aspects of carbohydrate fermentation by bacteria which still require elucidation. Even in the colon-typhoid group of bacteria, the representatives of which have been extensively studied, new, fundamental differences have been found to exist in the enzymic apparatus of the various species. These differences have hitherto been obscured by the presence of an accessory substance in the nutritive medium used. It is conceivable that some of the contradictory results regarding the ability of this or that organism to ferment a given carbohydrate or to produce gas, were due to the variable concentrations of accessory substances in different batches of media, different conditions of incubation or some other as yet undefined factor.

The data presented above show that not all the species of the colon-typhoid-dysentery bacteria are possessed of a complete enzymic apparatus for the utilization of sugar. The colon bacilli, *Eberthella typhosa* and *Salmonella paratyphi B.* can ferment glucose in a semi-synthetic medium which does not contain nicotinic acid. On the other hand, *Salmonella paratyphi A.* and the various strains of dysentery bacilli cannot ferment glucose in this medium unless nicotinic acid is added. Nutrient broth evidently contains nicotinic acid, because the addition of a small quantity of broth to our medium has the same effect as nicotinic acid. Another fundamental difference is thus found to exist between the two types of paratyphoid, A. and B. An additional simple means of differentiation is thus made available.

Of special interest is the observation of the effect of temperature on the further breakdown of lactic or some other acid in glucose fermentation. This phenomenon has been observed in the case of paratyphoid A and C, respectively, but not in the other strains of paratyphoid or in the coli strains tested.

We have not been able to elucidate the nature of the inhibition produced by temperature. The analyses indicate that with paratyphoid A. the failure to produce gas is accompanied by an

accumulation of lactic acid. It would seem, therefore, that the higher temperature inhibits the action of an enzyme, probably of the nature of a carboxylase. The fact that temperature can exert such an influence has apparently hitherto been overlooked.

Apart from the general interest these observations may have in relation to carbohydrate fermentation by bacteria, it may well be that the deterioration of the enzymic system of these organisms may have some bearing on their specific parasitism and invasiveness. The whole question requires re-investigation in the light of the newer knowledge of the relation of accessory substances to the physiologic activity of bacteria.

SUMMARY

Salmonella paratyphi A. and the various species of *Shigella dysenteriae* are unable to ferment glucose in semi-synthetic media which do not contain nictotinic acid. The Flexner strains are variable in this respect.

A high incubation temperature (37°C.) inhibits partially or wholly, according to the nature of the medium, the production of gas by *Salmonella paratyphi A.* and *C.* respectively, but not by other species of paratyphoid or coli bacilli tested.

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