The Behaviour of *Lactobacillus arabinosus* towards Nicotinic Acid and its Derivatives

By H. McILWAIN, D. A. STANLEY AND D. E. HUGHES
*Unit for Cell Metabolism (Medical Research Council), Department of Biochemistry,
University of Sheffield*

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These investigations originated in studying the basis of a method for microbiological assay of nicotinic acid (McIlwain & Stanley, 1948). The method was that in which acid produced from glucose by *Lactobacillus arabinosus* 17-5 is titrated with alkali after a period of incubation of about 3 days. The assay depends on the empirical observation that the quantity of titratable acid produced during incubation is dependent on the quantity of added nicotinic acid. It was found (McIlwain, 1948) that this relationship existed because of changes which took place in nicotinic acid during the assay; the compound was first assimilated to, and later lost from, the cells. Its loss was found to be conditioned by its functioning as a catalyst, and was such that nicotinic acid exhibited a defined catalytic capacity, measured by the ratio: titratable acid formed/nicotinic acid lost (in mol./mol.). Values of the catalytic capacity (about 5×10^6) were relatively independent of the intensity with which nicotinic acid derivatives were functioning as catalysts, which was expressed as a catalytic activity (in mol. titratable acid/hr./mol. nicotinic acid; values ranged from 5 to 15×10^3 hr.⁻¹).

Such observations suggested the need for more detailed investigations of the form in which nicotinic acid derivatives existed in the bacterial cells. We have now examined the nicotinic acid of cells of *Lb. arabinosus*, and found it to be largely in the form of cozymase. *Lb. arabinosus*, in marked distinction from a number of other organisms, reacted only slowly with added cozymase; this, and our interest in the practical problems presented by microbiological assay, directed our attention to metabolic changes occurring in the cozymase of the bacterial cells, and these are described below.

EXPERIMENTAL

The assays for nicotinic acid, the preparation of growth media and of materials for assay, and the handling of non-proliferating suspensions of *Lb. arabinosus* were carried out as described by McIlwain (1948). Determination of V-factor and of cozymase, and the extraction of these substances from bacteria were made by the methods used by McIlwain & Hughes (1948).

RESULTS

Assimilation of nicotinic acid by Lactobacillus arabinosus

1. *Cell content.* Cells of *Lb. arabinosus* from cultures whose growth has not been limited by nicotinic acid may, nevertheless, contain varying quantities of this substance. This is shown in Table 1. Nicotinic acid becomes a limiting factor in growth of the organism at concentrations of about 2×10^{-7} M and the range used in assay is below 1.5×10^{-7} M. The values of Table 1 illustrate also the previous findings (McIlwain, 1948) that the nicotinic acid content of the cells of a given culture falls with time. To approach saturation of the cells with the acid at 26 hr. in the relatively early part of a culture's growth, a concentration of c. 5×10^{-7} M-nicotinic acid is seen to be required. The cells will thus be unsaturated in this sense during most of their period of incubation in all the concentrations used in assay.

Under the conditions most favourable for assimilation, about 80 % of the nicotinic acid added to a culture has been recovered from the cells after 26 hr. growth. As the cells inactivate nicotinic acid during this period, this result gives a minimum estimate only of the completeness with which they can assimilate the acid. The actual concentration of nicotinic acid derivatives in the cells after assimilation can be calculated from the data of Table 1 to be about 2 to 10×10^{-4} M. In making this calculation the wet weight of the cells was taken as five times their dry weight; this has been found to be a typical ratio. The values recorded in Table 1 for the yield of bacterial cells from unit volume of culture show that the assay medium, though carefully chosen for maximum response to limited nicotinic acid, does not give high yields of bacteria.

2. *Nature of the assimilated nicotinic acid.* Most of the nicotinic acid of the cells existed as derivatives, especially in cells containing limiting quantities of the substance. Table 2 A shows that some 90 % of the acid, determined in cells grown from a concentration of the acid within assay range, exists as

Table 1. *Nicotinic acid content of cells of Lactobacillus arabinosus after growth of varying types*

Nicotinic acid of growth medium ($\mu\text{M} \times 10^{-7}$)	Period of incubation (hr.)	Yield of cells (mg. dry wt./ 100 ml. culture)	Nicotinic acid content of cells		
			($\mu\text{mol.}/\text{cells}$ of 100 ml. medium)	($\mu\text{mol.}/\text{mg.}$ dry wt.)	In fresh cells* ($\mu\text{M} \times 10^{-4}$)
1.2	30	7.0	6.2	0.88	1.8
2.4	26	10.4	13.9	1.33	2.7
4.9	26	9.5	37	4.04	8.1
4.9	42	10.7	33	3.06	7.1
4.9	48	12.0	28	2.34	4.7
12.2	26	9.8	53	5.4	10.8

* Calculated, assuming a water content of 80%.

Organisms were grown in quantities of 200–500 ml. of the assay medium (cf. McIlwain & Stanley, 1948) with nicotinic acid added in the quantities indicated.

Table 2. *Nicotinic acid derivatives in cells of Lactobacillus arabinosus, and in material liberated from cells to solution*

Nicotinic acid in growth medium ($\mu\mu\text{mol.}/100\text{ ml.}$)	Period of incubation in growth (hr.)	Yield of cells (mg. dry wt./ 100 ml. culture)	Later treatment	Content ($\mu\mu\text{mol.}/\text{cells}$ of 100 ml. culture)		
				Nicotinic acid	V-factor	Cozymase
A. Material in cells						
12.2	30	13.0	Washed	6.9	—	6.5
12.2	30	13.0	Washed, and in saline mixture* at 37°, 6 days	2.7	—	2.4
45	28	10.9	Washed	19	14	—
122	48	12.8	Washed	43	27	—
B. Material liberated on exposure of cells in salt mixture†						
45	28	10.9	Cells washed and	6.1	4.2	—
112	48	12.8	exposed†	16.4	8.4	—

* The inorganic salts of the medium of Barton-Wright (1946) at pH 6.8 and in the concentrations of the final assay medium.

† In the inorganic salts (organisms from 100 ml. of culture in 50 ml. salt solution) for 72 hr. at 37°, aerobically.

cozymase. The amount determinable as V-factor (probably equivalent to nicotinamide ribosides: for discussion see McIlwain, 1947) was also high.

3. *Relative stability of cozymase added to Lb. arabinosus.* McIlwain & Hughes (1948) found that many bacterial species rapidly inactivate added cozymase; even at low concentrations of cozymase ($c. 10^{-5}\text{ M}$) loss may occur at the rate of 10 $\mu\text{mol.}/\text{mg. dry wt.}/\text{hr.}$, and with higher concentrations may rise to over 500 $\mu\text{mol.}/\text{mg.}/\text{hr.}$ Such a coefficient means that the organism is inactivating its own weight of cozymase in about 3 hr. *Lb. arabinosus* was, however, relatively inert towards cozymase. Under the conditions of its use in assay, i.e. with cells of between 1 and 3 days growth, and at pH values between 5 and 7, any reaction with 10^{-5} or 10^{-4} M -cozymase was of $< 1\text{ } \mu\text{mol.}/\text{mg. dry wt. of cells}/\text{hr.}$ In attempts to observe a more rapid reaction, experiments were also carried out at pH 4.5 and 7.6, in the presence and absence of phosphates and of glucose, and with some suspensions initially rich and others initially poor in nicotinic acid derivatives. Again, any reaction was of $< 1\text{ } \mu\text{mol.}/\text{mg.}/\text{hr.}$ It was evident that *Lb. arabinosus* did not inactivate cozymase at the rapid rate observed in several other bacteria. Neverthe-

less, slow inactivation has been observed (Table 1), and found to be related to the assay (McIlwain, 1948); this process has, therefore, been studied further.

Change in nicotinic acid in suspensions of Lactobacillus arabinosus

An attempt was made to reproduce the changes of Table 1 under conditions better defined than those of a growing culture. Table 3 shows that the cellular nicotinic acid of *Lb. arabinosus* can also be very stable. Cells were examined which had been grown in media relatively rich and relatively poor in nicotinic acid; the latter conditions (12 $\mu\text{mol.}$ nicotinic acid/100 ml.) are within the range used in assay. In all cases the cells could be incubated at 37° in salt mixtures at pH 6.5–7 for 6 days without any loss in the total nicotinic acid of the suspension. A small increase, of some 10% of the initial value, was frequently found.

These experiments showed that the loss in nicotinic acid observed during the assay was not brought about by the bacteria when they were incubated in the presence of the majority of the constituents of the assay medium. The components omitted from the suspending fluids during the

experiments quoted were glucose, the casein hydrolysate providing amino-acids to the medium, and in one case sodium acetate. Other results in Table 3 showed sodium acetate to have only a small effect in the change in nicotinic acid; but addition of glucose caused a major change. In each case examined, addition of glucose led to a decrease in nicotinic acid content.

The course of changes in nicotinic acid derivatives in suspensions of Lactobacillus arabinosus

1. *In absence of glucose.* Although the total nicotinic acid of cell suspensions changed little under these conditions, marked changes occurred in its distribution. Fig. 1 shows this in the case of a suspension of cells which were initially rich in the

Table 3. *Changes in the nicotinic acid derivatives of suspensions of cells of Lactobacillus arabinosus*

Batch of organisms; conditions of growth	Initial content of nicotinic acid ($\mu\text{mol./mg. dry wt.}$)	Conditions of reaction	Change in nicotinic acid during 6 days* ($\mu\text{mol./mg. dry wt.}$)
1. 26 hr., 122 $\mu\text{mol.}$ nicotinic acid/100 ml.	5.4	Salts of medium,† pH 6.8	+0.70
		As above, with glucose (0.1M)	-1.80
2. 30 hr., 12 $\mu\text{mol.}$ nicotinic acid/100 ml.	0.88	Salts of medium, pH 6.5	+0.10
		As above, with glucose (0.1M)	-0.25
3. 26 hr., 24 $\mu\text{mol.}$ nicotinic acid/100 ml.	0.72	Inorganic salts of medium,‡ pH 7	+0.08
		As above, with glucose (0.1M)	-0.14

* The change quoted is in the whole reaction mixture; the kinetics of the change and the partition of nicotinic acid between cells and solution are discussed later.

† NaCl, Na acetate, and inorganic salts A and B of Barton-Wright (1946), at the concentrations used in assay.

‡ As in footnote †, but lacking Na acetate.

Fig. 1.

Fig. 2.

Figs. 1 and 2. Change in distribution of nicotinic acid in suspensions of cells of *Lb. arabinosus*, grown with 122 $\mu\text{mol.}$ of the acid/100 ml. Cells were harvested after 26 hr. growth, and washed with, and suspended in, the inorganic salts of the growth medium without glucose (Fig. 1) and with 0.1M-glucose (Fig. 2).

Although the effect of glucose was marked, the absolute magnitude of the change which it caused was small. It led to loss of about 30% of the cells' nicotinic acid in 6 days, but this involved an average rate of loss over that period of only 2-7 $\mu\text{mol./mg. dry wt. of cells/hr.}$ The change in a batch of cells grown from 12 $\mu\text{mol.}$ nicotinic acid/100 ml., and thus within the assay range, was about -2.5 $\mu\text{mol./mg./hr.}$, and thus much slower than that estimated as occurring during assay. This led us to investigate the course of the change, the distribution of nicotinic acid between cells and solution, and the effect of additional substances on the changes.

acid. The quantity in the cells fell throughout the experiment, but at a decreasing rate; and the acid which was lost from the cells appeared in the solution. Results similar to those of Fig. 1 have been obtained also with cells which were initially poorer in nicotinic acid (containing 30 or 15 $\mu\text{mol./100 ml.}$, the latter value being the upper limit of the assay range); but in such cases the proportion of nicotinic acid leaving the cells was smaller than with those initially rich in the acid.

The nicotinic acid-containing material liberated from the cells consisted at least in part of derivatives of the acid. The solutions yielded were too dilute to give significant values for cozymase in the apozy-

mase system, but their V-factor activity could be determined. The values obtained (Table 2B) showed two thirds or one half of the liberated material to possess such activity and presumably to be at least of the complexity of a riboside of nicotinamide.

2. *In presence of glucose.* Glucose led not only to the decrease in total nicotinic acid noted in the previous section, but in many cases it also caused

(1.22 $\mu\text{mol./10 ml.}$) within the assay range and were reaped at 30 hr. when their rate of acid formation was high. Their initial rate in the salts of the assay medium, with glucose, was $> 10 \mu\text{mol./10 ml./hr.}$, and this fell to about $1 \mu\text{mol./10 ml./hr.}$ on the sixth day. The rate fell rapidly during the first 2 or 3 days and then more slowly. Changes in the rate have, therefore, been examined in relation to changes in cellular nicotinic acid.

Table 4. *Effect of glucose on distribution of nicotinic acid in suspensions of Lactobacillus arabinosus*

Conditions of growth and reaction	Nicotinic acid (mμmol./10 ml. of suspension)			Percentage of nicotinic acid present in cells after 6 days
	Initially	After 6 days		
		Cells	Solution	
As 1, Table 3; no glucose	4.9	0.81	4.8	14
As 1, Table 3; with glucose	4.9	2.44	0.9	73
As 2, Table 3; no glucose	3.05	1.06	2.26	32
As 2, Table 3; with glucose	3.05	1.38	1.0	58
As 3, Table 3; no glucose	0.88	0.49	0.45	52
As 3, Table 3; with glucose	0.88	0.29	0.26	53

a marked change in the distribution of nicotinic acid derivatives between cell and solution. Fig. 2 shows that, with organisms rich in nicotinic acid, glucose caused nicotinic acid which otherwise would have passed into solution to be retained in the cells. The quantity in solution increased only transitorily, and then fell in a fashion roughly parallel to that of the cells. This behaviour was less marked in organisms which initially contained less nicotinic acid, and with cells grown from 12 $\mu\text{mol.}$ of the acid/100 ml. no considerable change in distribution of the acid was brought about by glucose (Table 4), but the fall in total quantity remained marked.

Acid formation from glucose by suspensions of Lactobacillus arabinosus

1. *Fall with time.* The changes in cellular nicotinic acid are relevant to the assay because this depends on acid formation by the cells, and this again depends on the presence of nicotinic acid derivatives. It has already been shown (McIlwain, 1948) that freshly harvested cells of *Lb. arabinosus* are capable of fermenting glucose at rates almost equal to the most rapid reached during growth in nutritionally rich media. Organisms from nicotinic acid-deficient cultures were found to produce acid at a lower rate than those with excess of the acid, and the deficiency could be made good by addition of nicotinic acid or cozymase. In these experiments acid formation was followed for only an hour or two after harvesting.

The course of acid formation from suspensions of *Lb. arabinosus* was now followed over periods comparable to those of the assay. Fig. 3 shows that observed in an experiment lasting 6 days. Organisms had been grown from a quantity of nicotinic acid

2. *Catalytic activity of nicotinic acid in cell suspensions.* Relevant results are presented in Table 5. Here the rate of acid formation in the suspensions is expressed as a multiple of the quantity of nicotinic acid in the cells. These values (catalytic activities: in mol. titratable acid/mol. nicotinic acid/hr.) are seen to be relatively constant but to fall during the course of exposure of the cells. The cells are thus changing

Fig. 3. Course of formation of titratable acid in suspensions of *Lb. arabinosus*, grown in the same way as the organisms of Figs. 1 and 2 but for 30 hr., and suspended in the medium described for Fig. 2.

during exposure so that nicotinic acid becomes progressively less effective as a catalyst. It is also initially much less effective as a catalyst during the present experiments than in assay; the catalytic activities of Table 5 are only about one tenth of those found (McIlwain, 1948) at comparable pH values during assay.

The higher catalytic activity during assay did not appear to be due to cell proliferation, as the earlier experiments included ones of short duration with harvested and resuspended cells. The difference was found to be due to the organic constituents of the suspending fluid. Reconstituting the assay medium by adding to the suspending fluid of Table 5 casein hydrolysate, ammonium salts, and a mixture of vitamin-like substances restored the catalytic activity to 14,000 mol./mol./hr. at pH 6, or 8000 at pH 5.2. In this effect the vitamin-like substances played little part (Table 5).

their cells. The assimilated material has been found to be present largely as cozymase, which is much more stable when added to suspensions of *Lb. arabinosus* than it is in suspensions of many other organisms. Loss of cozymase from buffered suspensions of *Lb. arabinosus* was extremely slow, both with respect to cozymase which was added as such, and also to that which was synthesized by the cells themselves from nicotinic acid. Cellular cozymase became markedly less stable in glycolyzing organisms although here also the rate of its loss was by most standards very low, being about $10 \mu\text{mol./mg. dry}$

Table 5. *Catalytic activity and catalytic capacity of nicotinic acid in acid formation by suspensions of Lactobacillus arabinosus*

(Cells were grown with $1.2 \mu\text{mol. nicotinic acid/10 ml.}$, reaped after 30 hr. incubation, washed twice aseptically with, and suspended in, the inorganic salts and buffers of the assay medium together with glucose but excluding the ammonium salts. Initial pH of medium 6.5; concentration of cells 2.95 mg./10 ml.)

Age of suspension (days)	pH	Nicotinic acid content of cells ($\mu\text{mol./10 ml.}$ suspension)	Rate of formation of titratable acid ($\mu\text{mol./10 ml.}$ suspension/hr.)	Catalytic activity (mol. titratable acid/ mol. nicotinic acid/hr.)	Catalytic capacity (mol. titratable acid formed/mol. nicotinic acid lost) ($\times 10^5$)
1	5.7	2.44	9.1	3700	3.5
2	5.4	2.04	4.6	2300	2.1
3	5.3	1.70	2.62	1500	2.3
4	5.25	1.48	2.04	1400	2.0
5	5.2	1.28	1.52	1200	2.1
6	5.2	1.14	1.08	1000	

A parallel experiment was carried out with similarly grown organisms in a suspending fluid of the same initial pH but with in addition the aneurin, riboflavin, pyridoxin, pantothenate, *p*-aminobenzoic acid and biotin of the assay medium. Catalytic activities of 2000–1200 were observed, and catalytic capacities of $2.5\text{--}3.5 \times 10^5$ (mean, 2.6×10^5).

3. *Catalytic capacity of nicotinic acid in cell suspensions.* In Table 5 are given also the ratios between the mol. titratable acid formed and the mol. nicotinic acid lost. These values (the catalytic capacities of nicotinic acid) were relatively steady during the experiments and were not far removed from the values observed in assay. Thus, different suspensions gave $2.5\text{--}3.5$ and $2.0\text{--}3.5 \times 10^5$, and in assay the capacity was about 5×10^5 . It has been observed above that in the present experiments, as in assay, loss of nicotinic acid was associated with the presence of glucose. The values now quoted show that the quantitative relations between the metabolism of glucose and of nicotinic acid are also similar during the assay in non-proliferating suspensions, although the absolute rates of metabolism of each fell in the suspensions to only a fraction of their value in assay.

DISCUSSION

The present studies have given further information on the behaviour of nicotinic acid in *Lactobacillus arabinosus*, and this behaviour may now be assessed in relation to the use of the organism in assay. The organisms, in growth, assimilated nicotinic acid from very dilute solutions and concentrated it in

wt./hr. It was, therefore, very interesting to observe that this rate bore a relation to the rate of glycolysis similar to that previously observed in more active cells during assay. In both cases the catalytic capacity of nicotinic acid, or the ratio between the rates of change in titratable acid in nicotinic acid, was between 2 and 6×10^5 . The possible significance of this has been discussed previously (McIlwain, 1948).

These values give another instance of relative stability in the catalytic capacity of nicotinic acid during marked changes in its catalytic activity (i.e. the rate of reaction which unit quantity of it catalyzes, in this case glycolysis, expressed in $\mu\text{mol./hr./}\mu\text{mol. nicotinic acid}$). The catalytic activity was markedly affected by the suspending fluid. This is understandable, as glycolysis involves many reactions, and the slowing of any one of these during the 6 days of an experiment could be reflected in the overall rate of reaction. The relative constancy of the catalytic capacity implies that, when glycolysis has fallen in rate, loss of nicotinic acid has also been correspondingly slower.

Data already available from various assay methods for nicotinic acid give further indications of the relative constancy of the catalytic capacity of the acid. As emphasized previously (McIlwain, 1948), the full catalytic capacity of nicotinic acid is

not used during an ordinary assay. In the instance investigated, normal assay practice, which allows reaction to continue until acid formation becomes slow, employs about half the full catalytic capacity of the added nicotinic acid. The standard dosage/response curve of assay, when expressed in mol. titratable acid/mol. nicotinic acid, thus gives a partial catalytic capacity of nicotinic acid. Values of this type have been collected in Table 6. This shows partial catalytic capacities from our own observations with *Lb. arabinosus* to range from 2.6 to 4.8×10^5 ; those of other observers with this organism, from 1.7 to 2.6 , and with *Leuconostoc mesenteroides*, from 4.9 to 6.8×10^5 . Fundamental importance is not

0.7 – 5 $\mu\text{mol./mg.}$ dry wt. of cells, according to conditions of growth.

2. The assimilated substance existed in the cells largely as cozymase. Cozymase in *Lb. arabinosus*, or added to suspensions of this organism, was much more stable than in the presence of many other bacteria.

3. A slow reaction (of 2 – 20 $\mu\text{mol./mg./hr.}$), leading to inactivation of cozymase and of its nicotinic acid moiety did, however, occur in *Lb. arabinosus* during glycolysis. Glycolysis altered also the distribution of nicotinic acid between saline solutions and the cells of the organism, leading to greater retention of the acid in the cells.

Table 6. *Partial catalytic capacities of nicotinic acid during assays*

Source of data	Conditions of assay		Partial catalytic capacity $\times 10^5$ *
	Organism	Temp. of incubation	
12 assays in conjunction with present paper, and that of McIlwain (1948)	<i>Lactobacillus arabinosus</i>	37	2.6–4.8; mean, 3.4; standard deviation, 0.65
Barton-Wright (1946)	" "	30 or 37	
Snell & Wright (1941)	" "	30	
Roberts & Snell (1946)	" "	37	
Krehl, Strong & Elvehjem (1943)	<i>Leuconostoc mesenteroides</i>	30	4.9
Johnson (1945)	" "	30	6.75

* Molar ratio: titratable acid formed/nicotinic acid added, during the approximately linear portion of the dosage/response curve. Different media of growth were employed by the different investigators; titratable acid was in all cases determined after 3 days' growth.

attributed to the partial catalytic capacities which are expressed immediately by these values, but the latter are considered to indicate that the catalytic capacities themselves are also likely to be of the same order of magnitude under the various conditions chosen by the different observers whose results have been given in Table 6. This increases the significance of the catalytic capacity.

SUMMARY

1. *Lactobacillus arabinosus* 17–5, which is used in a microbiological assay of nicotinic acid, assimilated this acid from 10^{-8} or 10^{-7} M solutions during growth. Its cell content of the substance was then

4. Rates of glycolysis and of loss in nicotinic acid varied in parallel in suspensions of *Lb. arabinosus*, as they had previously been found to do in assay. The ratio between the two rates (the catalytic capacity of nicotinic acid) had approximately the same value, of 3 – 6×10^5 , in the two cases.

5. On the other hand, the activity of nicotinic acid as a catalyst was much lower in non-proliferating suspensions in simple media, than in assay. Catalytic activity and partial catalytic capacity have been used as measures of the behaviour of the organism during assays of the present type.

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