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Transport of B-Vitamins in Microorganisms

VII. The Uptake of ^{14}C-Niacinamide by Non-proliferating Cells and by Protoplasts of *Streptococcus faecalis*

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Non-proliferating cells of *Streptococcus faecalis* were incubated with labelled niacinamide under varying conditions and the radioactivity retained by the carefully washed cells was measured.

Selected uptake studies were, in a similar way, carried out with protoplasts prepared by lysozyme treatment.

Extracts from the labelled cells and protoplasts were subjected to paper chromatography. Although most of the labelled niacinamide was rapidly after the uptake converted to other compounds, estimations of the intracellular and extracellular concentrations indicated that the accumulation of niacinamide proceeds against considerable concentration gradients. The uptake of niacinamide in both, non-proliferating cells and in protoplasts, exhibited also several other characteristics of active transport, *viz.* dependence on pH and on a supply of metabolic energy, as well as saturation kinetics.

The accumulation capacity of the non-proliferating cells with respect to labelled niacinamide varied inversely with the niacinamide content of the growth medium.

The uptake in protoplasts was considerably affected by K^+, Mg^{2+}, and Ca^{2+}, whereas the uptake in intact cells was much less sensitive to these agents.

It was reported previously that the uptake of labelled thiamine by non-proliferating cells of *L. fermenti* exhibited many characteristics of active transport, *i.e.* dependence on a metabolic energy source, pH and temperature optima, stereospecificity and accumulation of the vitamin against a concentration gradient.[1,2] The corresponding accumulation in cells of organisms normally not requiring exogeneously supplied thiamine was independent of a metabolic energy source and, in most cases studied, insignificant.[3]

Streptococcus faecalis, a niacin requiring organism, was selected for the studies on the transport of this vitamin, because methods have been described for the preparation of protoplasts and cell membranes from this organism.[5]

The present paper deals with studies on the uptake of labelled niacinamide by non-proliferating cells and by protoplasts of *Streptococcus faecalis*.

MATERIALS AND METHODS

Labelled niacinamide (carbonyl-^{14}C) was obtained from the Radiochemical Centre, Amersham, England. *Streptococcus faecalis* (ATCC 9790) was grown in media of a composition similar to that described earlier for *L. fermenti*,[1] but containing 1 mg thiamine per litre and variable levels of niacinamide. Washed cell suspensions were incubated with ^{14}C-niacinamide (carbonyl-^{14}C) directly and/or after conversion to protoplasts.

Cell and protoplast suspensions. The preparation of the washed cell suspensions was carried out essentially as previously described.[1] Protoplasts were prepared by lysozyme treatment as introduced by Weibull [4] for *B. megatherium* and mainly as described by Abrams *et al.*[5] for *S. faecalis*, but employing 0.4—0.6 M sucrose containing 0.15 M Tris-HCl pH 6.5. The suspensions of protoplasts contained less than 1 % of viable cells as determined by standard plating technique. The protoplasts were washed once with the sucrose-Tris-HCl medium and re-suspended in the same medium, in a volume equal to the volume of the original washed cell suspension.

Uptake experiments. The incubation of the washed cell suspensions with the labelled vitamin was carried out essentially using methods previously described.[1] The suspensions of protoplasts (2 ml) were added to the sucrose-Tris-HCl medium (3 ml) containing variable components. After completed incubation with the labelled vitamin, carried out in a manner analogous to that employed for the washed cell suspensions, the protoplast-containing tubes were cooled to 4°C and the protoplasts were spun down and washed three times with the sucrose-Tris-HCl medium. The radioactivity of the sedimented protoplasts was determined by the liquid scintillation method [6] after digestion with formamide.

The uptake experiments comprised studies on the influence of pH, incubation time, cell ("enzyme") and niacinamide ("substrate") concentration, exogeneous energy source, metabolic inhibitors and certain ions.

Chromatographic studies. Extracts for chromatography from both cells and protoplasts were prepared as previously described for cells of *L. fermenti*.[2] Chromatographic studies were carried out on extracts from cells and protoplasts in order to establish if, for how long a time and to what extent the niacinamide taken up remained free or was converted to some other compounds. The radioactive spots were localized by means of autoradiography and evaluated quantitatively by the liquid scintillation method as described elsewhere.[2] The relationship between the niacin content of the growth medium and the ability of the nonproliferating cells to take up niacinamide was also investigated.

RESULTS AND DISCUSSION

The optimum pH was found to be in the range 5.5—7.0 in the presence of potassium phosphate and in the range 6.0—7.5 in the presence of sodium phosphate. Analogously to what has been previously found with respect to the uptake of thiamine in *L. fermenti* [1] the uptake of niacinamide in *S. faecalis* was considerably higher when potassium phosphate was used as buffer than when sodium phosphate was employed. At pH 4.5, employing either potassium or sodium acetate buffers, the uptake was practically non-existent, whereas it increased rapidly in the range pH 4.5—5.5, much more so with potassium than with sodium acetate buffer. At pH 5.5 the uptake in the presence of potassium acetate was considerably higher than in the presence of potassium phosphate. This might have been due either to the better buffering capacity of acetate at this pH as compared to phosphate, or to preferential use (in the presence of potassium?) of acetate as an energy source instead of glucose. For subsequent experiments the pH-value of 6.5 was selected.

The uptake of labelled niacinamide was proportional to the amount of cells up to 3—5 mg cells (dry solid material) per ml. Results of time-uptake studies indicated that the reaction was essentially complete after 60 min and that

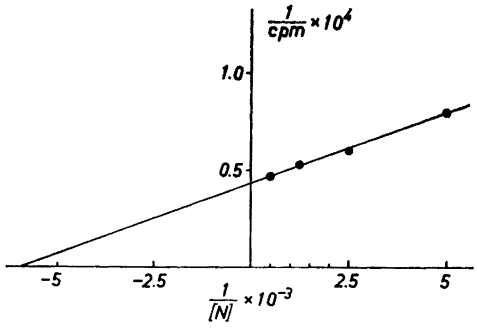

Fig. 1. The Lineweaver-Burk plot of reciprocal uptake velocity (v = cpm taken up in 20 min by 10^8 cells) *versus* the reciprocal molar concentration of exogeneous niacinamide (N). Niacin deficient non-proliferating cells of *S. faecalis* exposed to ^{14}C-niacinamide. Incubation mixture: potassium phosphate 0.02 M pH 6.5, glucose 0.06 M, sodium chloride 0.15 M, ^{14}C-niacinamide 0—8 × 10^{-3} M. Incubation at 37°C.

more than 80 % of the total uptake (in intact cells) had already accumulated after 20 min of incubation. The corresponding value for protoplasts varied somewhat from experiment to experiment. Incubation for 20 min was selected for subsequent experiments. The relationship between the exogeneous concentration of niacinamide and the uptake indicated saturation kinetics as defined by the Michaelis-Menten theory for enzyme mediated reactions (*cf.* Fig. 1). From the Lineweaver-Burk plot of reciprocal uptake velocity versus the reciprocal concentration of exogeneous niacinamide, given in Fig. 1, the K_m for the uptake reaction under the conditions of the test, can be calculated to be approximately 1.7 × 10^{-4} mM niacinamide.

The accumulation capacity of washed cell suspensions with respect to ^{14}C-niacinamide varied inversely with the content of niacinamide (or niacin) in the growth medium over a wide range of concentrations as shown in Table 1. This is analogous to the previously observed relationship between the content of thiamine in the growth medium and the accumulation capacity of washed

Table 1. The effect of the niacinamide content of the growth medium on the ability of washed cell suspensions of *S. faecalis* to accumulate ^{14}C-niacinamide. Incubation conditions as in legend to Fig. 1.

Content of niacinamide in the growth medium μg/ml	Radioactivity taken up by 2 × 10^8 cells cpm	Notes
100	585	
10	562	
1	2180	"normal" cells
0.1	11171	
0.01	22156	"niacin deficient" cells
0.001	15750	

cells of *L. fermenti* with respect to ^{14}C-thiamine.[1] However, at growth limiting concentrations of niacinamide (1 mμg per ml) the accumulation capacity of the washed cells decreased (*cf.* Table 1). This could be explained by some damage to the transporting apparatus resulting from the severe niacin deficiency. The cells obtained from a medium containing 1 μg niacinamide per ml were considered as "normal", whereas the cells obtained from a medium containing 0.01 μg of the vitamin per ml were considered as "niacin deficient".

Results of studies on the influence of glucose, iodoacetate and certain ions on the uptake of ^{14}C-niacinamide by intact cells and by protoplasts of *S. faecalis* are summarized in Table 2.

Table 2. The influence of certain compounds on the radioactivity accumulation by washed cell suspensions and by protoplasts of *S. faecalis* after exposure to ^{14}C-niacinamide. Incubation mixture: Tris-HCl 0.15 M pH 6.5; sucrose 0.4—0.6 M (only in the case of protoplasts); sodium chloride 0.15 M (only in the case of cells); ^{14}C-(carbonyl-^{14}C) niacinamide 0.2—0.4 × 10^{-6} M; 10^{8} cells/ml; metal ions added as chlorides. Incubation for 20 min at 37°C. Results compiled from several experiments.

Further additions to the incubation mixture,	M	Radioactivity taken up by Normal Cells %	Normal Protoplasts %	Deficient Cells %	Deficient Protoplasts %
None		0.5—3.0	7—15	0.5—3.0	14—45
Glucose	0.06	100	100	100	100
Glucose + iodoacetate	0.06 0.1	0	0	0	0
Glucose + Mg^{2+}	0.06 0.02	80—100	250—300	100—110	160—250
Glucose + Ca^{2+}	0.06 0.02			100	20
Glucose + K^{+}	0.06 0.002	*a*	14	*a*	24
Glucose + K^{+}	0.06 0.004	*a*	6	*a*	13
Glucose + Na^{+}	0.06 0.002	*b*	125	*b*	90
Glucose + Na^{+}	0.06 0.004	*b*	131	*b*	100

[a] K^{+} at concentrations 0.02—0.15 M stimulated to some extent the uptake by intact cells (*cf.* the discussion of the pH effect above).

[b] 0.15 M NaCl was employed in the suspension medium for uptake studies with intact cells.

It can be seen that exogeneous glucose increased considerably the uptake of niacinamide by both the cells and the protoplasts and that this glucose effect was, in both cases, entirely inhibited by iodoacetate. Whereas the uptake in intact cells in the absence of exogeneous glucose was only a few percent of the corresponding uptake when glucose was supplied, the uptake in protoplasts was considerable even in the absence of added glucose, *viz.* 15—45 % of the uptake with glucose in protoplasts prepared from deficient cells and 7—15 % in protoplasts obtained from normal cells. The effects of Mg^{2+} and Ca^{2+} were, at least at the concentrations tested, insignificant in the case of intact cells. The uptake in protoplasts, however, was stimulated by the addition of Mg^{2+} and inhibited by the addition of Ca^{2+}.

An inhibiting effect on the accumulation of niacinamide in protoplasts was also observed with small concentrations of K^+ (0.002—0.004 M), whereas corresponding concentrations of Na^+ were essentially without effect or possibly slightly stimulating.

It is interesting to note in this connection reports by Abrams [7,8] according to which protoplasts of *S. faecalis* undergo a reversible metabolic swelling as a result of glycolysis (0.01 M glucose) in the presence of K^+ (0.002—0.01 M), whereas Na^+ ions have no such effect and rather inhibit the effect of K^+ without affecting the rate of glycolysis. The degree of the reversibility of the K^+-induced swelling increased in the experiments of Abrams [7] with the molarity of sucrose (employed as the osmotic stabilizer) and decreased with the concentration of glucose (0.01—0.04 M), the maximum swelling taking place after approximately 20—30 min of incubation. The concentration of glucose employed in our experiments was higher (0.06 M) and the uptake of niacinamide decreased in the presence of K^+, whereas the K^+-induced reversible swelling in the experiments of Abrams was followed by an increase in the protoplast permeability. This indicates that, under our experimental conditions, the presence of K^+ probably induced an irreversible swelling of the protoplasts with a concomitant lysis.

Results of chromatographic studies employing 77 % aqueous isopropyl alcohol and 80 % aqueous ethyl alcohol indicated that most of the radioactivity taken up is rapidly converted to other compounds, moving on the chromatograms more slowly than niacinamide. After only 2 min of incubation with the labelled vitamin no more than 5—15 % of the total radioactivity of the protoplast extracts were present as free niacinamide. This figure remained essentially the same after extended times of incubation (up to 120 min). The corresponding figure obtained with intact cells was higher, *viz.* 20—25 % of free niacinamide in the extracts from cells exposed to the labelled vitamin during 2—20 min and possibly less than that after incubation for 20—120 min.

Estimations of concentration gradients, analogous to those previously reported for the uptake of labelled thiamine in *L. fermenti* [2] indicated that the ratio between the "intracellular" and extracellular concentrations of niacinamide was, after 20 min of exposure to the labelled vitamin, at least 3000:1 as calculated on the basis of total uptake. When only the free "intracellular" niacinamide was taken into consideration the corresponding ratios were approximately 600:1 and 300:1 in the case of intact cells and protoplasts, respectively. The calculations of concentration gradients were based on the

estimations of cell weight (from dry solid contents and microscopic cell number determinations) and on the assumption that the cell water constitutes approximately 80 % of the cell mass. (For further details, *cf.* Ref. 2, Discussion).

From the results obtained so far it seems that the uptake of niacinamide in non-proliferating cells and in protoplasts of *S. faecalis* exhibits several characteristics of active transport, *viz.* dependence on pH and on a supply of metabolic energy, saturation kinetics, and accumulation against a concentration gradient.

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