

Transport of B-Vitamins in Microorganisms

VIII. Comparative Studies on Membrane Bound ATPase(s) Obtained from Normal and Niacin Deficient Cells of *S. faecalis*

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Cytoplasmic membranes were prepared from normal and niacin deficient cells of *S. faecalis* by direct lysis of the intact cells in the presence of lysozyme. The P_i releasing activities of the membrane preparations were studied using ATP and certain other phosphate containing substrates.

Both kinds of preparation contained Mg^{2+} -activated ATPase which was, at least in the preparations from normal cells, further stimulated by certain concentrations of K^+ and Na^+ . The activation by Mg^{2+} was lowered in the presence of Ca^{2+} .

Comparative studies on 18 preparations from normal and 18 preparations from niacin deficient cells indicated that the specific ATPase activity of the membranes was considerably depressed in niacin deficiency.

The membrane material also contained inorganic pyrophosphatase, the specific activity of which was, in preparations from normal cells, equal to that of the ATPase, whereas it was lower than the specific ATPase activity in membranes from niacin deficient cells.

The membrane preparations released approximately ten times more P_i from ATP than they did in the presence of ADP; AMP was not hydrolyzed by the preparations.

The data accumulated in the literature indicate that membrane bound ATPases, stimulated by Na^+ and K^+ , from a variety of animal tissues may be involved in the phenomenon of active transport (*cf.* a review by Hokin and Hokin¹). It was found in this laboratory that the uptake of niacin by non-proliferating cells of *S. faecalis* exhibited several characteristics of active transport.² Similar observations were made with respect to the uptake of thiamine in non-proliferating cells of *L. fermenti*.^{3,4}

Results of previous studies indicated that the ATP-hydrolyzing activities of cell fractions from *L. fermenti* were depressed when the cells were grown under conditions of thiamine deficiency.⁵ At the same time, the thiamine deficient non-proliferating cells exhibited much greater capacity to accumulate thiamine than the non-proliferating cells grown in media satisfying all the

nutritional requirements of the organism.³ An analogous relationship with respect to the uptake of niacinamide was observed with cells of *S. faecalis* grown in normal and niacin deficient media, respectively.² It was therefore considered to be of interest to compare the ATPase activities of normal and niacin deficient cells of this organism. Abrams *et al.*⁶ have prepared protoplast membranes ("ghosts") from *S. faecalis* and demonstrated that most of the ATPase activity of this organism is membrane bound.* These authors suggested that the membrane ATPase may be involved in bringing about changes in the permeability of the cell towards, *e.g.*, certain oligosaccharides. The present studies were therefore confined to the membrane bound ATPase(s) from normal and niacin deficient cells of *S. faecalis*.

EXPERIMENTAL PROCEDURE

Reagents. Adenosine-5'-triphosphate disodium salt (ATP), adenosine-5'-diphosphate sodium salt (ADP), and adenosine-5'-monophosphate sodium salt (AMP) were preparations from Sigma, St. Louis, Mo., USA, as were also DNAase and lysozyme. Sodium pyrophosphate reagent grade, was purchased from Merck, Darmstadt, Germany. All other chemicals were also of reagent grade. Re-distilled water was used throughout the investigation.

Organism and growth media. Details of the maintenance, storage and cultivation of *Streptococcus faecalis* (ATCC 9790) were similar to those previously described for *L. fermenti*.³ For the preparation of protoplast membranes the cells were grown in media of a composition similar to that given in the last mentioned work, but containing 1 mg thiamine per litre and variable levels of niacinamide. Cells grown in media containing 1 µg niacinamide per ml were designated as normal, whereas those grown at 0.01 µg/ml were considered as niacin deficient.² Preparation of the two kinds of cells was usually carried out in two steps involving 2 × 5 ml and 100 ml cultures, respectively. Incubation (37°C) during each step was allowed to proceed for 20 h, after which time the cells were in the stationary phase of growth. According to Abrams⁷ cells of *S. faecalis* taken during the logarithmic phase of growth do not give stable protoplasts. This indicates that the membranes from such cells may not be fully developed.

Preparation of protoplast membranes. The cells obtained by centrifugation of the growth medium were washed three times with re-distilled water and re-suspended in a buffer solution to give a density corresponding to 5% transmission using Coleman Spectrophotometer, Model 14. In the initial experiments the protoplast membranes were prepared by osmotic lysis of protoplasts obtained by lysozyme treatment of the washed cells suspended in 0.4 M sucrose-0.15 M Tris-HCl buffer, pH 6.4, essentially as described by Abrams *et al.*⁶ The membrane suspensions resulting from this procedure could not be obtained in a homogeneous form, however, owing to the presence in the suspension of large viscous particle aggregates. This rendered an accurate quantitation of the membrane material for protein and enzyme determinations very difficult. An improvement was obtained by adopting the procedure of Bibb and Straughn⁸ who prepared membranes from a *Streptococcus* strain by direct lysis of the intact cells in the presence of deoxyribonuclease to decrease the viscosity of the particle aggregates. The final procedure adopted in the experiments reported here was as follows: The washed cells were incubated for 2 h at 37°C in Tris-HCl buffer, 0.1 M, pH 7.0, containing MgSO₄ 0.01 M, lysozyme 200 µg/ml and deoxyribonuclease 1 µg/ml. The mixture was stirred magnetically (low speed) in order to keep the cell material in suspension. The resulting membranes were sedimented in Spinco Preparative Ultracentrifuge, Model L 50, at 38 000 g for 15 min. The sediment was repeatedly washed with 0.1 M Tris-HCl buffer

* According to a recent report by Abrams¹⁸ the membrane bound ATPase from *S. faecalis* can be released into solution if the membranes are repeatedly washed with water instead of 10⁻³ M MgSO₄ as was done previously.⁶

pH 7.0 containing 0.01 M MgSO_4 . The washings were carried out by re-suspending the sediment in the buffer and transferring it carefully to a stoppered vial in which it was vigorously shaken with a few glass beads. The homogeneous suspension was then transferred back to the centrifuge tube and sedimented again as above. The procedure was repeated until the protein content of the supernatant was only approximately 20 $\mu\text{g}/\text{ml}$. This level was usually obtained in the third supernatant and it remained essentially the same after several subsequent washings thus indicating that some of the proteins normally associated with the membranes may dissolve upon extended washing. A number of four successive washings was selected as a standard procedure for the experiments reported here. No ATPase activity was found in the washings. The protein content of the final membrane suspensions was determined by the method of Lowry *et al.*⁹ employing 1 M NaOH. The membrane suspensions could be stored for several days at $+4^\circ\text{C}$ without any significant decrease in the ATPase activity. Occasionally, a certain increase in the enzyme activity was observed upon storage. In one case enzyme assays carried out after one and twenty days of storage respectively, gave essentially the same values. Usually, the membrane suspensions were tested for ATPase activity within 2–3 days.

Enzyme assays. Unless otherwise stated the reaction mixture contained Tris-HCl buffer 0.1 M, pH 7.5, ATP disodium salt 5×10^{-3} M, MgSO_4 2.5×10^{-3} M, membrane protein 125 $\mu\text{g}/\text{ml}$, total volume 2 ml. The reaction was started by the addition of the ATP solution and stopped (usually after 20 min at 37°C) by the addition of 1 ml ice-cold 1 M perchloric acid. Further details were as previously described.⁵ The enzyme preparations to be compared were always analyzed simultaneously. The protein level was kept constant within each experiment and in most experiments it was kept at 0.125 mg/ml incubation mixture. Every incubation set contained substrate blanks and three levels of inorganic phosphate to check the standard curve. Enzyme blanks were tested in certain experiments, but never gave any phosphate reaction. Every determination was carried out in duplicate and every experiment repeated at least once. The nucleotide products of the reaction were determined by descending paper chromatography on Whatman No. 1 paper for 18 h at 22°C in isobutyric acid:ammonia: $\text{H}_2\text{O} = 50:1:20$ as described by von der Decken.¹⁰ The spots were localized by means of UV-illumination.

The comparative ATPase studies on membrane material from normal and niacin deficient cells were carried out in such a way that one normal culture always was grown simultaneously with one niacin deficient culture and the corresponding membrane preparations were tested simultaneously for their ATP-hydrolyzing activities.

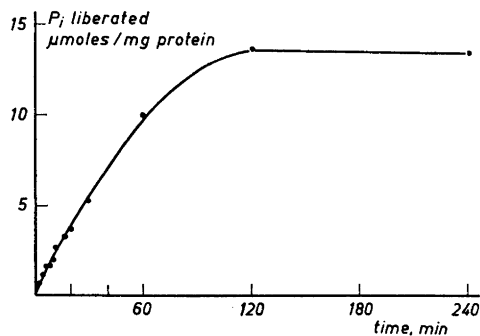


Fig. 1. The relationship between the amount of liberated inorganic phosphate (P_i) and the incubation time. Incubation at 37°C in a total volume of 2 ml containing 5×10^{-3} M ATP, 2.5×10^{-3} M MgSO_4 , 0.1 M Tris-HCl, pH 7.5, 0.25 mg membrane protein (*S. faecalis*).

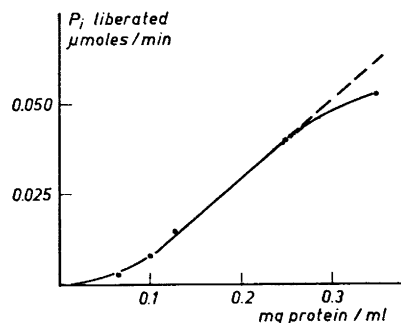


Fig. 2. The relationship between the amount of liberated inorganic phosphate (P_i) and the amount of membrane protein (*S. faecalis*) incubated with ATP. Incubation as in Fig. 1 but for 20 min.

RESULTS

Unless otherwise stated the data shown in Figs. 1–5 as well as the discussion of the kinetic properties of the enzyme refer to preparations from normal cells.

The course of the liberation of inorganic phosphate (P_i) upon incubation of the membrane material with ATP for varying lengths of time is shown in Fig. 1. It can be seen that there is a linear relationship between the amount of P_i liberated and time during the first 60 min. An incubation time of 20 min was selected for a standard procedure as representing the initial reaction velocity. This was found to be valid in the case of membrane preparations from normal as well as from deficient cells. The reaction velocity varied linearly with the amount of the membrane material in the incubation mixture in the range 0.1–0.25 mg protein/ml (*cf.* Fig. 2). In most experiments reported here a concentration of 0.125 mg of membrane protein/ml incubation mixture was employed.

The optimum ratio of Mg^{2+} :ATP concentrations was studied in a number of experiments using membranes from both normal and niacin deficient cells and was invariably found in the range 1:4–1:2, rather close to 1:2. Results of a representative experiment are shown in Fig. 3 (*cf.* also Fig. 4). Since the membrane material contained some residual Mg^{2+} derived from the washings, there was some ATP hydrolyzing activity (20–25 % of the maximum) even in the absence of added Mg^{2+} . It is obvious, however, that Mg^{2+} was required for the ATPase activity of the membrane material. Ca^{2+} was inhibitory at all the Mg^{2+} concentrations tested. Increasing concentrations of Ca^{2+} had a gradually increasing inhibition effect at a given concentration of Mg^{2+} . When the concentration of Mg^{2+} was varied equimolarly with that of Ca^{2+} the degree of the inhibition by Ca^{2+} was essentially the same at all the concentrations of Ca^{2+} that were tested (*cf.* Fig. 4). Results of chromatographic studies indicated that the only nucleotide formed during the reaction was ADP. This was observed with preparations from normal as well as with preparations from niacin deficient cells.

Results of studies on the stimulation of the Mg^{2+} -activated ATPase by K^+ and Na^+ are shown in Fig. 5 by means of a representative experiment. The concentration of NaCl in the incubation mixture was kept constant at

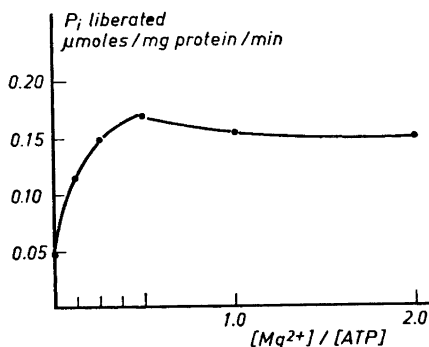


Fig. 3. The influence of the ratio between the concentrations of Mg^{2+} and ATP on the amount of liberated inorganic phosphate (P_i) during incubation of membrane material (*S. faecalis*) with ATP. Incubation (20 min) as in Fig. 1 except for $MgSO_4$ which was varied between $0-10^{-2}$ M.

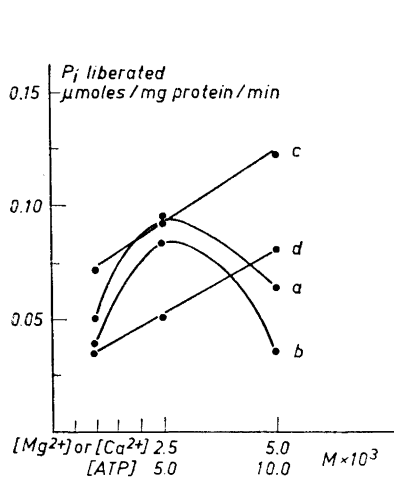


Fig. 4. The influence of Ca^{2+} on the Mg^{2+} -activated liberation of inorganic phosphate (P_i) upon incubation of membrane material from *S. faecalis* with ATP. Incubation for 20 min at $37^\circ C$ in a total volume of 2 ml containing 0.1 M Tris-HCl pH 7.5, variable ATP, $MgSO_4$ and/or $CaCl_2$; a: ATP constant 5×10^{-3} M, $MgSO_4$ variable 10^{-3} – 5×10^{-3} M; b: as a, but with $CaCl_2$ equimolar with $MgSO_4$; c: ATP variable 2×10^{-3} – 10^{-2} M, constant Mg^{2+} :ATP ratio = 1:2; d: as c, but with $CaCl_2$ equimolar with $MgSO_4$; a–b and c–d were carried out with two different preparations.

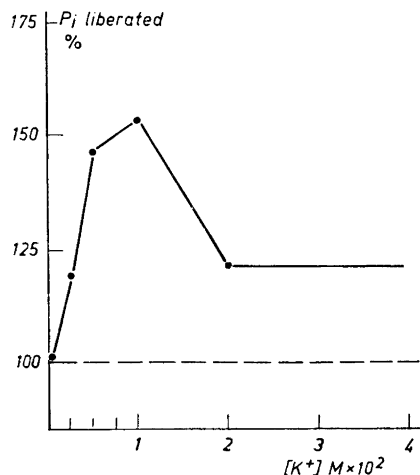


Fig. 5. The stimulation by K^+ – Na^+ of the Mg^{2+} -activated ATPase present in membrane material from *S. faecalis*. Incubation (20 min) as in Fig. 1, but in the presence of 0.1 M NaCl (except for the dashed line which denotes the P_i -releasing activity in the absence of monovalent cations, taken as 100%) and in the presence of varying amounts of KCl (0–0.04 M).

0.1 M and the concentration of KCl was varied between 0 and 0.04 M. It can be seen in Fig. 5 that, under these conditions, the largest stimulation was obtained with 0.01 M K^+ , *i.e.* when the ratio between the concentrations of K^+ and Na^+ was approximately 1:10. Such stimulation by K^+ – Na^+ was observed with seven out of nine preparations from normal cells that were tested and in one out of two tested preparations from niacin deficient cells. The stimulation usually amounted to 10–80% of the activity in the absence of the monovalent cations. Na^+ alone (0.1 M) had practically no stimulating effect or possibly a very slight one (*cf.* Fig. 5).

The P_i -releasing activities of 18 membrane preparations from normal and 18 simultaneously tested membrane preparations from niacin deficient cells of *S. faecalis* are listed in Table 1. The preparations from normal cells liberated on the average 0.19 ± 0.06 (range 0.08–0.31) μ moles inorganic phosphate per mg membrane protein and min, whereas the corresponding average activity of the preparations from niacin deficient cells was 0.13 ± 0.05 (range 0.07–0.21). The preparations from the deficient cells, thus exhibited, on the average, 69 ± 20 % (range 41–95%) of the ATP-hydrolyzing activity of the preparations from normal cells.

As can be seen in Table 1 there were considerable variations within each group of values. However, each value obtained with a preparation from normal cells was higher than the value obtained with the simultaneously tested preparation from niacin deficient cells.

Table 1. The P_i -releasing activities of membrane preparations from normal (N) and niacin deficient (D) cells of *S. faecalis*. Incubation as in Fig. 1, but for 20 min.

Preparation No.	P_i liberated by		D % of N
	N μ moles	D P_i /mg protein/min	
1	0.31	0.16	52
2	0.24	0.21	88
3	0.27	0.19	70
4	0.13	0.10	77
5	0.20	0.14	70
6	0.20	0.17	85
7	0.23	0.10	43
8	0.19	0.07	37
9	0.22	0.09	41
10	0.12	0.06	50
11	0.20	0.19	95
12	0.18	0.15	83
13	0.18	0.15	83
13	0.18	0.15	83
14	0.14	0.12	86
15	0.16	0.14	88
16	0.08	0.07	88
17	0.14	0.07	50
18	0.26	0.13	50
Mean	0.19	0.13	69
Standard deviation	0.06	0.05	20

Table 2. The average P_i releasing activities of membrane preparations from normal (N) and niacin deficient (D) cells of *S. faecalis* using various phosphorylated compounds. Incubation conditions as in Fig. 1, but for 20 min. The data represent initial reaction velocities. Numbers in brackets refer to the number of respective preparations that were tested.

Substrate 5×10^{-3} M	P_i liberated by		D % of N
	N μ moles	D P_i /mg protein/min	
ATP ^a (18)	0.19 \pm 0.06	0.13 \pm 0.05	69 \pm 20
ADP (3)	0.024 \pm 0.010	0.015 \pm 0.007	62 \pm 12
AMP (3)	0	0	
PP _i (4)	0.36 \pm 0.18	0.09 \pm 0.04	26 \pm 8
» corrected ^b	0.18 \pm 0.10	0.04 \pm 0.02	26 \pm 9

^a from Table 1.

^b corrected with respect to the reaction: PP_i \rightarrow 2P_i.

The average P_i -releasing activities of membrane preparations from normal and niacin deficient cells using various phosphorylated compounds are shown in Table 2. It can be seen that both kinds of preparation liberated approximately ten times less inorganic phosphate in the presence of ADP than they did with ATP as substrate and that there was no phosphate liberation in the presence of AMP. The P_i releasing activities with respect to ADP of the preparations from the deficient cells were, on the average, 62 ± 12 % of the corresponding activities of the preparations from normal cells. A considerable hydrolysis of inorganic pyrophosphate (PP_i) took place with both kinds of preparation, but, in this case, the activity of the "deficient" preparations was only 26 ± 9 % of the corresponding activity of the "normal" preparations. Further, whereas the membranes from normal cells were virtually equally efficient with PP_i and with ATP as substrate, the membranes from deficient cells were only approximately 30 % as efficient with PP_i as with ATP.

DISCUSSION

In agreement with the observations of Abrams *et al.*⁶ the membrane material from *S. faecalis* obtained in our experiments by a different procedure contains an Mg^{2+} -activated ATPase which removes the terminal phosphate group from ATP, liberates much less inorganic phosphate from ADP than from ATP and does not or essentially does not utilize AMP as substrate. There are, however, certain differences in other properties of the respective enzyme preparations. We have found the optimum Mg^{2+} :ATP ratio in the reaction mixture to be rather close to 1:2 whereas the aforementioned authors have reported the ratio 1:1 to give maximum ATPase activity. The membrane material prepared in our experiments contained, in addition to the ATPase, also considerable amounts of inorganic pyrophosphatase with a specific activity as high as that of the ATPase. Only very small amounts of P_i were liberated from inorganic pyrophosphate by the preparations of Abrams *et al.*⁶ These differences may depend on the different procedures employed for the preparation of membranes as well as on the entirely different growth media used by us as compared to the growth media used by Abrams and his co-workers.^{6,11} Our results with membrane preparations from cells grown in media deficient in niacin indicate that the composition of the growth medium may have a profound influence on both the ATPase and the pyrophosphatase activities of such preparations.

It can be seen in Table 2 that the ATPase activity of membrane preparations from niacin deficient cells is approximately 70 % of that found in preparations from normal cells, whereas the pyrophosphatase activity of the "deficient" preparations is only approximately 22 % of that of the "normal" ones. Thus, it seems that, *e.g.*, low contents of niacin in a medium rich in all other nutrients depresses the pyrophosphatase activity of the membranes to a much higher degree than is the case with the ATPase activity. It cannot be concluded, however, from the results of this investigation whether, in niacin deficiency, the pyrophosphatase was more depressed or more extensively released from the membranes than was the ATPase. It is interesting to note in this connection the results of previous studies with *L. fermenti* which indi-

cated that, in thiamine deficiency, not only were the ATPase activities of the soluble and particulate cell fractions depressed, but also the distribution of the ATPase between these fractions was changed in favour of the soluble enzyme.⁵

In addition to the results obtained by Abrams *et al.*⁶ with their preparations of *S. faecalis* membranes, also the results of Weibull *et al.*¹² with membranes of *B. megatherium* as well as previous results obtained in this laboratory with particulate and soluble enzymes from *L. fermenti*⁵ indicate that inorganic pyrophosphatase activity resides mainly in the soluble cell fractions of these organisms. It was therefore surprising to find such high pyrophosphatase activities in our membrane preparations from *S. faecalis*. The DNAase preparation employed in the present experiments (but not in the experiments quoted above) to reduce the viscosity of the membrane containing material was found essentially inactive with inorganic pyrophosphate.

The Mg^{2+} -activation of the membrane bound ATPase was, in our experiments, inhibited by Ca^{2+} . This is analogous to what has been reported for membrane bound ATPases from animal tissues^{13,16} and to what has been previously observed with ATPase(s) from *L. fermenti*.⁵ Contrarily to the enzyme(s) from this organism, however, the ATPase in *S. faecalis* membranes was not activated by Ca^{2+} *per se*. This was possibly due to the presence in the enzyme preparations of residual Mg^{2+} derived from the washings of the membranes (*cf.* Fig. 3). The amount of the ATPase inhibition by Ca^{2+} in the presence of Mg^{2+} was essentially the same with Ca^{2+} -concentrations varying between 10^{-3} – 10^{-2} M provided that the concentrations of Ca^{2+} and Mg^{2+} were kept at an equimolar level (*cf.* Fig. 4). With a constant Mg^{2+} -concentration in the incubation mixture, however, increasing Ca^{2+} -concentrations caused a gradually increasing inhibition.

It seems interesting that the Mg^{2+} -activated ATPase in our membrane preparations was further stimulated by $K^+ - Na^+$. This is analogous to what has been reported for a number of membrane bound ATPases from animal tissues (for review *cf.* Ref. 1) after the original discovery by Skou¹³ of the ATPase stimulation by $K^+ - Na^+$. This author was also the first to suggest that such Mg^{2+} -activated $K^+ - Na^+$ stimulated ATPases may be linked to the active transport of the monovalent cations.¹³⁻¹⁵ The extent of the ATPase stimulation by $K^+ - Na^+$ seems to vary with the different enzyme sources studied.¹ According to Skou¹³⁻¹⁵ the extent of the $K^+ - Na^+$ stimulation is related to the concentrations of other cations present in the incubation mixture. The largest stimulation by $K^+ - Na^+$ was, under our experimental conditions, observed when the $K^+ : Na^+$ ratio was 1:10. This correlates fairly well with certain literature data for animal enzyme systems (*cf.*, *e.g.*, Refs. 13, 14, 16, 17). The extent of the stimulation by $K^+ - Na^+$ of the Mg^{2+} -activated ATPase in membranes of *S. faecalis* (10–80 %) seems to be of the same order of magnitude as similar effects reported for certain animal enzyme systems (*cf.*, *e.g.*, Ref. 16), but it appears lower than has been reported for certain other preparations from animal tissues (*cf.*, *e.g.*, Ref. 17).

Although ATP disodium salt was used in most of our experiments to study the influence of K^+ in the presence of Na^+ the sodium amount added with the substrate did not interfere, since the molarity of the Na^+ derived from

the ATP preparation was only 0.01 M (5×10^{-3} M ATP), whereas that of the NaCl in the incubation mixture was 0.1 M (*cf.* Fig. 5).

In addition to the observation that the membrane bound Mg^{2+} -activated ATPase from *S. faecalis* is further stimulated by $K^+ - Na^+$ the results of this investigation indicate also that the specific activity of the ATPase is depressed when the cells are grown under conditions of niacin deficiency. The depression, however, is not selective with respect to the ATPase since also the hydrolysis of PP_i seems to be lower using membranes from niacin deficient cells as compared to preparations from normal cells. The specific pyrophosphatase activity is twice as much depressed as is the ATPase. It is therefore difficult, from the results of this investigation, to find a specific correlation between the observed depression of ATPase activity and the increased capacity of the niacin deficient cells to accumulate labelled niacin.²

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