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THE EXTRACELLULAR NUCLEASE ACTIVITY OF *MICROCOCCUS SODONENSIS*

III. KINETIC STUDIES AND CONTROL OF PRODUCTION*

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SUMMARY

1. Kinetic analyses of the purified nuclease of *Micrococcus sodonensis* yielded K_m values of $2.9 \cdot 10^{-6}$ and $2.83 \cdot 10^{-5}$ M for RNA and AMP, respectively.

2. AMP was inhibitory to ribonuclease activity and apparent non-competitive kinetics were obtained with a K_i of $2.9 \cdot 10^{-3}$ M.

3. Both diesterase and monoesterase activities were stimulated by chelating agents such as glycine, histidine and EDTA, and by sulfhydryl compounds such as mercaptoethanol, cysteine and GSH. *p*-Chloromercuribenzoate was inhibitory to both activities as were certain of the purine nucleotide analogues such as inosine 5′-chloromethylphosphonate.

4. Studies with metabolite indicated that enzyme synthesis was controlled by the intracellular pyrimidine nucleotide concentration. A cytidine nucleotide is implicated.

5. The data presented in the paper add additional support to the "one protein–two activities" concept for this enzyme.

INTRODUCTION

The isolation and purification of the extracellular nuclease from *M. sodonensis* has been previously reported[1]. This enzyme is released into the medium by logarithmically growing cells and its production was shown to be NH_3 dependent. Two activities were found associated with the pure protein[2], a depolymerase active on

Abbreviation: PCMB, *p*-chloromercuribenzoate.

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RNA, DNA, poly A and adenylyl-3',5'-adenosine, and a 5'-nucleotidase, and evidence was presented for the existence of the two activities on a single protein molecule, involving two active sites, one of which is shared by both monoesterase and diesterase activity. The concept of a single protein with two activities is not unique and several such enzymes have been described in the literature[3–6].

This report deals with the kinetic analyses and inhibitor studies of the purified enzyme as well as the demonstration that the production of this extracellular enzyme or its precursor form is controlled by the intracellular nucleotide pool.

The physical and chemical characterization of the enzyme and the involvement of the cell wall in its synthesis are discussed in the subsequent paper.

MATERIALS AND METHODS

Organism and culture conditions

M. sodonensis ATCC 11880 was employed in these studies. Cells were grown at 30°, with vigorous aeration either in trypticase soy broth (Baltimore Biological Laboratories) for enzyme isolation, or in the synthetic medium of CAMPBELL *et al.*[7] for control studies. Cell growth was determined turbidometrically and converted to dry weight of washed cells by means of a previously prepared standard curve. Cell-free extracts were prepared from 18-h cultures of *M. sodonensis* grown in synthetic medium *plus* NH_4^+. Cells were washed and disrupted in a Hughes press, and resuspended in appropriate buffer to yield a protein concentration of about 5 mg/ml.

Enzyme purification

Supernatant liquid from 18–24-h cultures of *M. sodonensis* were concentrated and dialysed by ultrafiltration using an Amicon Model 400 cell and a Diaflo XM-50 membrane. Samples were filtered under nitrogen at 50 lb/inch^2. Following dialysis of the concentrate, the DEAE-cellulose, gel filtration sequence previously described[1] was employed except that Biogel P-200 was used instead of Sephadex G-200 for the gel filtration step.

Materials

RNA (A grade) and the purine and pyrimidine nucleosides and nucleotides used were obtained from Calbiochem. ^{14}C-Labeled RNA was obtained from Schwartz BioResearch Inc. Lithium salts of inosine chloromethylphosphonate and inosine fluorophosphonate were the kind gift of Dr. A. Hampton, Cancer Research Institute, University of Alberta. Reagents for polyacrylamide gel electrophoresis were purchased from Eastman Organic Chemicals.

Enzyme assays

(a) Diesterase activity. Standard reaction mixtures contained: 2 mg RNA, 33 μmoles Tris, 13 μmoles $MgCl_2$, 1.7 μmoles $CaCl_2$, 1.7 μmoles $MnCl_2$, 10 μmoles mercaptoethanol and 1 μmole EDTA per 1.9 ml. Final pH of the mixture was 8.8. 0.1 ml of enzyme was added at zero time, the reaction incubated at 37°, and removed at the designated times and added to equal volumes of cold 0.2% uranyl acetate in 10% trichloroacetic acid. Activity was followed either by the release of

acid-soluble 260-mμ absorbing material, or by the release of acid-soluble radioactivity from ^{14}C-labeled RNA. Radioactivity was determined by means of a Nuclear Chicago Mark I scintillation counter. 1 unit of activity is defined as the amount of enzyme which releases 1 μmole of acid-soluble nucleotide per h.

(b) Monoesterase activity. The standard reaction mixture was identical to that for diesterase except that AMP (2 μmoles) was the substrate employed. After incubation at 37° the reaction was stopped by the addition of an equal volume of cold 0.2% uranyl acetate in 10% trichloro-acetic acid and the amount of P_i released was determined by the colorimetric procedure of AMES AND DUBIN[8].

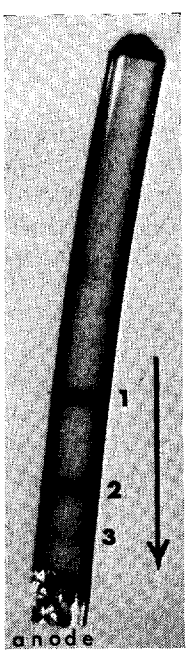
Gel electrophoresis

The method of DAVIS[9] was employed. Electrophoresis was carried out using Tris–glycine buffer (pH 8.6), and a current of 2 mA/tube. Protein was detected by staining the gel in 1% amido black in 7% acetic acid for 1 h. Excess stain was removed in 7% acetic acid using the rapid destaining apparatus (Metaloglass Inc.) with a current of 70 mA/tube.

RESULTS

Gel electrophoresis

Fig. 1 shows the results obtained when a partially purified (post-DEAE chromatography) nuclease preparation was subjected to polyacrylamide gel electro-



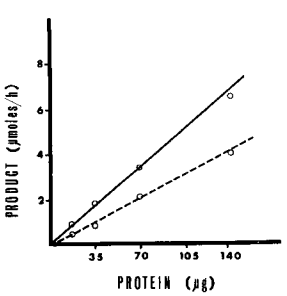


Fig. 1. Polyacrylamide gel electrophoresis of a partially purified preparation of *M. sodonensis* nuclease. Protein was stained with 1% amido black in 7% acetic acid. Monoesterase and diesterase activities were located in band No. 3.

Fig. 2. Effect of enzyme concentration on reaction rate of *M. sodonensis* nuclease. ○—○, dieste -rase activity; ○— — —○, monoesterase activity.

phoresis for 2 h at 2 mA/tube. Three bands of protein were detected which had migrated towards the anode. Duplicate gels were sliced into 1-mm segments, eluted in 0.1 M Tris for 1 h at 25° and assayed for monoesterase and diesterase activity as described in MATERIALS AND METHODS. Both activities were located in band No. 3. Gel electrophoresis of purified enzyme also showed the two activities in a single band at concentrations where no band of protein was clearly demonstrable by the amido black technique.

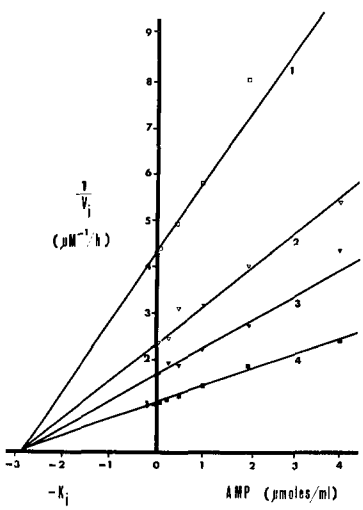
Kinetic analyses

The effect of enzyme concentration on activity was investigated. Fig. 2 shows the results obtained when varying concentrations of enzyme protein were added to reaction mixtures with AMP and RNA as substrates. The expected linear relationship was obtained indicating that reaction velocity is proportional to enzyme concentration.

K_m values were determined for both AMP and RNA. Reaction velocities were calculated at several concentrations and the K_m values obtained from Lineweaver–Burk plots of the data were $2.83 \cdot 10^{-5}$ M for AMP and $2.9 \cdot 10^{-6}$ M for RNA (or 1.45 mg/ml as weight of RNA). The value for RNA was based on an assumed molecular weight of $5 \cdot 10^5$, calculated from a sedimentation constant of 16 S.

Competition experiments

The effect of AMP on diesterase activity was determined at several RNA



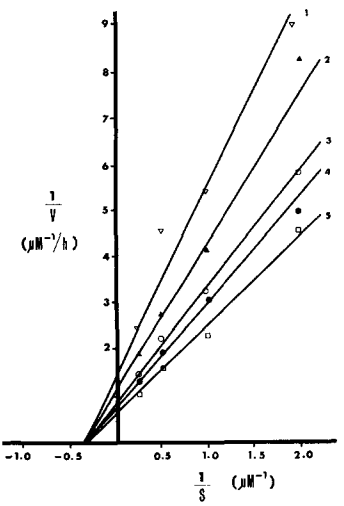


Fig. 3. Graphical determination of the inhibitory effect of AMP on diesterase activity of *M. sodonensis* nuclease. Assays were carried out as described in MATERIALS AND METHODS and the release of label soluble in 0.2% uranyl acetate in 10% trichloroacetic acid from varying concentrations of ^{14}C-labeled RNA in presence of varying amounts of AMP was determined. Concentrations of RNA were as follows: Curve 1, 0.25 mg/ml; Curve 2, 0.5 mg/ml; Curve 3, 1.0 mg/ml; Curve 4, 2.0 mg/ml.

Fig. 4. Lineweaver–Burk plot of diesterase activity of *M. sodonensis* nuclease in the presence of AMP. Activity was followed as described in Fig. 3. Concentrations of AMP were as follows: Curve 1, 4 μmoles/ml; Curve 2, 2 μmoles/ml; Curve 3, 1 μmole/ml; Curve 4, 0.5 μmole/ml; Curve 5, control no AMP.

concentrations. ^{14}C-Labeled RNA was added (0.125 μC/mg) and the release of acid-soluble radioactivity was measured in the presence of varying amounts of non-radioactive AMP. Plotting inhibitor (AMP) concentration *vs.* the reciprocal of the velocity (Fig. 3) yielded a K_i for AMP of $2.9 \cdot 10^{-3}$ M. A Lineweaver–Burk plot of the same data (Fig. 4) gave apparent non-competitive inhibition kinetics in that the v_{max} was altered but the K_m was unchanged in the presence of AMP. This was confirmed by the fact that the slope of the v/v_i *vs.* inhibitor concentration curve was independent of substrate concentration. The validity of this interpretation is discussed later.

Experiments were carried out to compare the total velocity (v_t) in the presence of both substrates with the velocities obtained with each substrate alone. Since the

TABLE I

COMPARISON OF THE "MIXED SUBSTRATE" VELOCITY WITH "SINGLE SUBSTRATE" VELOCITIES OF *M. sodonensis* NUCLEASE

RNA concentration (mg/ml)	*AMP concentration (μmoles/ml)*	*Velocity (μmoles P_i released per h)*
2	—	1.455
2	2	1.03
1	—	0.963
1	2	0.87
—	2	0.457

products of both reactions[2] are nucleosides *plus* P_i, the assay of P_i was considered valid for total velocity. Table I shows the results obtained with digests containing 1 and 2 mg/ml of RNA in the presence and absence of 2 μmoles of AMP. As indicated in the table, the total velocity in the mixture with the two substrates falls between the two values obtained with RNA and AMP separately.

Enzyme activation

A stimulatory effect on both activities was obtained by chelating agents, for

TABLE II

EFFECT OF EDTA ON ACTIVITY OF *M. sodonensis* NUCLEASE

EDTA concentration (M)*	*Relative activity*	
	Monoesterase	*Diesterase*
0	100	100
$1 \cdot 10^{-2}$	50.8	50
$1 \cdot 10^{-3}$	104	70
$1 \cdot 10^{-4}$	132	80
$1 \cdot 10^{-5}$	140	102
$1 \cdot 10^{-6}$	150	130

* Final concentration in assay mixture.

example, 0.1 M glycine, 0.1 M histidine, and EDTA. The stimulation was considered to be non-specific and a result of the removal of traces of toxic heavy metals. As shown in Table II, inhibition occurs at higher levels of EDTA where essential ions such as Mg^{2+}, Mn^{2+} and Ca^{2+} would be chelated, however at low concentrations, heavy metals such as Fe^{2+}, Hg^{2+}, Cu^{2+}, *etc*, which have a greater affinity for EDTA, would be preferentially bound.

Sulfhydryl compounds also were shown to be stimulatory and Table III shows the effect of the addition of several such compounds. Since mercaptoethanol exhibited

TABLE III

ACTIVATION OF *M. sodonensis* NUCLEASE BY SULFUR-CONTAINING COMPOUNDS

Addition	*Concentration* (M)*	*Relative activity*	
		Monoesterase	*Diesterase*
None	—	100	100
Mercaptoethanol	0.01	500	200
Cysteine	0.01	230	184
GSH	0.01	270	146

* Final concentration in assay mixture.

the greatest effect on both activities, it was routinely added to the assay mixtures to yield a final concentration of 0.01 M.

Enzyme inhibition

Since physical methods have failed to separate monoesterase and diesterase activities it was hoped that inhibitor studies might be of value. If two proteins were involved it should be possible to obtain a selective inhibition of one activity and to separate the enzyme–inhibitor complex thus formed. Two types of inhibitors were employed: (a) those which act on the enzyme itself (*e.g. p*-chloromercuribenzoate (PCMB)) and (b) nucleotide analogues which would compete with the substrate for the active site of the enzyme.

TABLE IV

INHIBITION OF ACTIVITY OF *M. sodonensis* NUCLEASE BY PCMB

PCMB concentration (M)*	*Relative activity*	
	Monoesterase	*Diesterase*
0	100	100
$1.25 \cdot 10^{-4}$	87	95.7
$2.5 \cdot 10^{-4}$	73.8	82.4
$5.0 \cdot 10^{-4}$	58	74.3
$1.0 \cdot 10^{-3}$	46	61.5
$2.0 \cdot 10^{-3}$	31	48

* Final concentration in assay mixture.

Table IV shows the results of an experiment in which PCMB was added to the reaction mixture in the concentrations indicated. Reactions were carried out in the absence of mercaptoethanol and EDTA. Inhibition of both activities occurred although the effect was slightly greater on the monoesterase than the diesterase activity.

Several nucleotide analogues were employed, which were altered at various sites on the molecule: inosine 5'-chloromethylphosphonate, inosine 5'-fluorophosphonate, adenosine 5'-monoacetate, adenosine 5'-phosphomorpholidate, and 6-azauridine 5'-monophosphate. Neither of the adenosine analogues was effective as either inhibitor or substrate, however both inosine analogs were inhibitory to the same extent. Table V shows the effect of inosine 5'-fluorophosphonate on both activities. As can be seen from the table, inhibition of both activities occurred although in the presence of substrate the inhibition was less marked.

TABLE V

EFFECT OF INOSINE FLUOROPHOSPHONATE ON ACTIVITY OF *M. sodonensis* NUCLEASE

Condition	*Analogue concentration* (μmoles/ml)*	*Relative activity*	
		Monoesterase	*Diesterase*
No preincubation	0	100	100
	2	56	69
	4	56	66
Preincubated	0	100	100
	2	58	33
	4	19	10

* Concentration in the final assay mixture. IMP (1 μmole/ml) and RNA (1 mg/ml) were employed as substrates. Preincubation of enzyme and analogue was carried out overnight at room temperature in the absence of substrate.

Table VI shows the effect of 6-azauridine 5'-monophosphate on both activities in the presence of substrate. There appeared to be little, if any, effect on the diesterase activity and the effect on the monoesterase was less than that exerted by the inosine

TABLE VI

EFFECT OF 6-AZUARIDINE 5'-MONOPHOSPHATE ON ACTIVITY OF *M. sodonensis* NUCLEASE

Concentration (μmoles/ml)*	*Relative activity*	
	Monoesterase	*Diesterase*
0	100	100
0.66	90	98
1.33	74.2	95.9
2.7	62.2	94.29

* Final concentration in assay mixture.

analogues. This is perhaps not unexpected since the affinity of the enzyme for pyrimidine nucleotides is less than that for purine nucleotides[2].

Ribose 5-phosphate and ribose 1-phosphate were also employed, but neither would serve as the substrate nor as inhibitor in the system.

Control of production

The requirement for NH_3 for growth and enzyme production of *M. sodonensis* and its possible role in carbamyl phosphate synthesis has been discussed previously[1,7]. The stimulatory effect of NH_3 is most probably not due to NH_3 *per se* but rather to some intracellular intermediate whose synthesis is NH_3 dependent, consequently the systems in *M. sodonensis* involving NH_3 were investigated to yield possible

TABLE VII

EFFECT OF CYTIDINE AND NH_4^+ ON NUCLEASE ACTIVITY OF *M. sodonensis* CULTURE SUPERNATANTS

Assay	*Medium*	*Activity (units/mg dry cell wt.)*					
		12 h	*16 h*	*24 h*	*30 h*	*36 h*	*48 h*
Diesterase	Synthetic	—	—	—	—	—	—
	Synthetic + NH_4^+						
	10 μmoles/ml	—	0.171	0.88	0.835	0.76	0.72
	1.0 μmoles/ml	—	—	—	—	—	0.3
	Synthetic + cytidine						
	10 μmoles/ml	—	—	—	—	0.37	0.445
	1.0 μmoles/ml	—	—	—	—	—	0.207
Monoesterase	Synthetic	—	—	—	—	—	—
	Synthetic + NH_4^+						
	10 μmoles/ml	—	0.58	1.565	1.234	0.791	0.628
	1.0 μmoles/ml	—	—	—	—	0.314	0.543
	Synthetic + cytidine						
	10 μmoles/ml	—	—	—	0.311	0.752	1.55
	1.0 μmoles/ml	—	—	—	—	—	0.614

clues as to the potential control substance. The experiments were designed solely to delineate the possibilities open to the cell and are qualitative only. (For details as to procedure and results, see ref. 11.) The following systems were qualitatively established as existing in cell-free extracts:

Carbamoylphosphate synthase, aspartate carbamoyltransferase, ornithine carbamoyltransferase, CTPsynthetase, GMP synthetase, glutaminase, an inducible asparaginase, and cytidine deaminase.

With a knowledge of the existing systems, one could then select products which might be active in control of enzyme synthesis. None of the amino acids tested (aspartate, glutamate, arginine, ornithine, citrulline) had any effect on enzyme production. Glutamine could replace NH_4^+ in the synthetic medium but this was found to be due to the very active glutaminase which is present constitutively in the organism. There was also an inducible asparaginase present and therefore asparagine, after a long lag, could produce a similar effect[11].

All the available ribo- and deoxyribonucleosides were tested and only cytidine

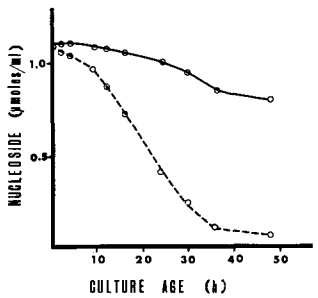


Fig. 5. Fate of exogenous cytidine in *M. sodonensis* culture supernatants. *M. sodonensis* was grown in a synthetic medium containing 1 μmole/ml of cytidine. Aliquots were removed and centrifuged and the supernatant diluted in 0.2 M HCl. Loss of cytidine was determined by measuring absorption at 290 mμ. Loss of nucleoside was followed by measuring absorption at 267 mμ. ○—○, nucleoside; ○— — —○, cytidine.

and deoxycytidine derepressed enzyme production. Table VII compares the effect of cytidine with that of NH_4^+ on diesterase and monoesterase activities. As can be seen from the data, cytidine produces the same effect as low levels of NH_4^+ in that there is a lag before enzyme is produced.

The supernatants from the above experiment were examined spectrophotometrically to determine the fate of exogenous cytidine. Fig. 5 shows the results obtained when 1.0 μmole/ml of cytidine was added to the medium. By 48 h the cytidine was completely deaminated to form uridine while only about 0.3 μmole of

TABLE VIII

EFFECT OF URIDINE AND NH_4^+ ON NUCLEASE ACTIVITY OF *M. sodonensis* CULTURE SUPERNATANTS

Assay	*Addition**	*Activity (units/mg dry cell wt.)*				
		15 h	*24 h*	*30 h*	*35 h*	*48 h*
Diesterase	None	—	—	—	—	—
	Uridine	—	—	—	—	—
	NH_4^+		0.184	0.202	0.196	0.170
	NH_4^+ + uridine	0.205	0.256	0.268		0.243
Monoesterase	None	—	—	—	—	—
	Uridine	—	—	—	—	—
	NH_4^+		0.19	0.416	0.352	0.395
	NH_4^+ + uridine	0.406	0.566	0.602	0.68	0.86

* Uridine was added to yield a final concentration of 10 μmoles/ml. NH_4^+ was added to yield a final concentration of 1 μmole/ml.

nucleoside per ml were taken up by the cells. The effect of cytidine, therefore, could have been due either to the nucleoside itself or to the NH_3 released by the deaminase (as was the case with glutamine) and used in the synthesis of some other compound. If the cytidine itself were involved then one should be able to see an additive affect of uridine in the presence of sub-optimal levels of NH_4^+. Table VIII shows the results obtained from one such experiment. Although uridine itself did not stimulate

TABLE IX

EFFECT OF PHOSPHATE ON NUCLEASE PRODUCTION BY *M. sodonensis*

Phosphate concentration (M)	*Relative activity*	
	Diesterase	*Monoesterase*
$3.17 \cdot 10^{-4}$	100	100
$6.34 \cdot 10^{-4}$	44.9	43.9
$1.58 \cdot 10^{-3}$	15	12.6
$3.17 \cdot 10^{-3}$	6	5
$6.34 \cdot 10^{-3}$	0	0

enzyme synthesis, when it was added to sub-optimal levels of NH_4^+ there was much higher activity than with NH_4^+ alone at the same concentration.

Enzyme synthesis could also be repressed markedly by increasing the phosphate concentration in the medium above the level required for optimal growth. The growth curve was not affected but as shown in Table IX there was a marked inhibitory effect on nuclease production and at a concentration of 20 times that required for optimal growth no activity could be detected.

DISCUSSION

As discussed previously[2] the nuclease of *M. sodonensis* is homogenous by all the techniques employed and attempts to separate diesterase from monoesterase activity have been unsuccessful. The data obtained in the present study support rather than dispute the "one protein–two activities" hypothesis.

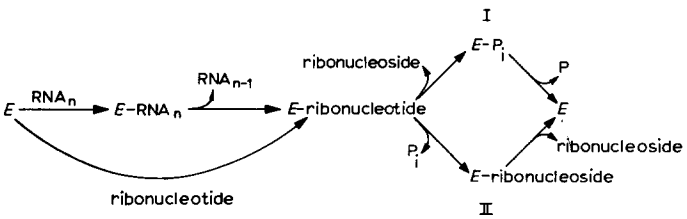
Selective inhibition of one activity could not be obtained without a similar and concurrent inhibition of the other. Both activities are stimulated by the addition of sulfhydryl compounds and are inhibited by PCMB. The stimulatory and inhibitory effects are more pronounced on the monoesterase than the diesterase but this can be explained, if the site uniquely involved in binding mononucleotide has a sulfhydryl group on or in close proximity to it. The same relative stimulation effected by chelating agents adds further support to this concept since they would prevent the formation of mercaptides with the essential sulfhydryl group by toxic heavy metals.

When a single enzyme is active on two substrates A and B, the total velocity (v_t) in a reaction mixture containing both substrates will fall between the two velocities which would be obtained with A and B alone at the same concentrations[12]. If two proteins are involved then v_t would be equal to the sum of the two individual velocities. This was confirmed by STURGE AND WHITTAKER[13] with horse serum cholinesterase when they showed that the rate of hydrolysis of benzoyl choline alone was 440 μl/h, that of isoamyl acetate alone was 131 μl/h and in a mixture of the two, the rate was 314 μl/h (determined manometrically). They concluded from these data that the hydrolysis of the choline and non-choline esters was due to the same enzyme. A similar situation exists for *M. sodonensis* nuclease where the v_t for RNA *plus* AMP falls between v_{RNA} and v_{AMP} thus strengthening the evidence for a single protein.

DIXON AND WEBB[12] state that when an enzyme protein is active on more

than one substrate that each substrate will behave as a competitive inhibitor for the other. The Lineweaver–Burk plot obtained with *M. sodonensis* nuclease (Fig. 4) suggests a non-competitive inhibition of diesterase activity by AMP, a surprising result since both AMP and RNA are substrates. However, apparent non-competitive inhibition kinetics in the initial stages of a reaction can be obtained between one substrate and another competing for a single site when the affinity for one substrate is significantly higher than for the other. This was demonstrated by MYERS[14] with an inhibitor of cholinesterase (Nu-683) which had a very high affinity for the enzyme. Although the inhibitor was in fact competitive, it exhibited apparent non-competitive kinetics. The interpretation of the Lineweaver–Burk plot is based upon the model of one active site and where the substrates differ markedly in size, the kinetics of the reaction must be exceedingly complex and preclude a straightforward interpretation of the plot.

A suggested mechanism of reaction is as follows:



Either Pathway I or II is possible in the conversion of E–NMP to $E + N + P_i$ and a more detailed kinetic analysis is underway to further elucidate the steps of the reaction.

The studies with analogues permit conclusions to be drawn concerning the sites of attachment to the substrate. The requirement of a purine or pyrimidine on C-1 is evident since although 5′-nucleotides are substrates for the enzyme, ribose 5-phosphate is neither substrate nor inhibitor for either activity. There is no requirement for a free 2′-hydroxyl group on the sugar moiety since DNA and the deoxyribonucleotides will serve as substrates. The 3′-hydroxyl is essential and if blocked in any way the activity is inhibited. Although pTpT will serve as a substrate, thymidine 3′,5′-diphosphate will not[2], nor will cyclic 3′,5′-AMP[2]. It was also shown that borate, which forms a complex between the 2′- and 3′-hydroxyl groups on the substrate, inhibited activity (S. A. BERRY, unpublished data). An ionizable group at the 5′ position is essential since adenosine 5′-monoacetate will not function either as a substrate or as an inhibitor whereas inosine 5′-fluorophosphonate and inosine 5′-chloromethylphosphonate are inhibitors for both activities. Although adenosine 5′-phosphomorpholidate has an ionizable group it is not bound, which is likely a result of steric hindrance by the large 5′ substituent group. It can be concluded that at least three sites on the substrate molecule are required for enzyme activity; (a) a purine or pyrimidine base; (b) a free 3′-hydroxyl; (c) an ionizable 5′ group.

It has been shown[11] that when *M. sodonensis* is grown in a complex medium, such as Trypticase soy broth, nuclease production is initiated during the logarithmic phase and the amount produced per unit cell remains constant. In a synthetic medium, containing NH_4^+ however, the amount of enzyme per unit cell increases

until early in the stationary phase after which it decreases slightly. It was suggested that this increased synthesis was in response to an accumulation of some intracellular intermediate. In medium containing sub-optimal levels of NH_4^+ enzyme production was delayed and activity could not be demonstrated until cells were entering the stationary phase. Since cell yield at the time of enzyme appearance was considerably higher in the low NH_4^+ medium than in the normal medium it would appear that the accumulation of an intracellular inducer rather than a depletion of nutrients is responsible for the initiation of enzyme synthesis. In the optimal medium, ample NH_4^+ is present for all NH_3-dependent systems and the intracellular level of "inducer" could build up rapidly. However, when NH_4^+ is limiting, more time is required to bring the "inducer" to a level at which it will initiate enzyme synthesis.

The demonstration of an NH_3-dependent carbamoylphosphate synthase strongly suggested that a pyrimidine nucleotide was implicated as the active derepressor and this was confirmed by the whole-cell studies. With the addition of cytidine to the medium the growth eventually reached the level of that in the normal synthetic medium *plus* NH_4^+. The appearance of enzyme was delayed analogous to the situation in the low NH_4^+ medium, again indicating the requirement for sufficient levels of intracellular "inducer" with the deamination of cytidine and the uptake of the pyrimidine being rate limiting. As Fig. 5 demonstrates, the uptake of the nucleoside occurs at a much slower rate than the deamination of cytidine. Uridine alone had no effect on either growth or enzyme production but in the presence of sub-optimal levels of NH_4^+ it effected a significant increase in both monoesterase and diesterase production and enzyme appeared much earlier in the growth cycle than when cytidine or low levels of NH_4^+ alone were present. These results suggest that cytidine is the active derepressor but that it must first be deaminated to uridine in order to be taken up by the cell, then reconverted to cytidine intracellularly. The amination of uridine compounds has been demonstrated in this organism.

Since the intracellular pool is at the nucleotide level it is likely that CMP rather than cytidine is the controlling factor. The repression of synthesis by increased levels of phosphate is also indicative of the involvement of a nucleotide since such an excess of phosphate produces an imbalance of the nucleotide pool. Confirmation of the nature of the repressor and derepressor involves the quantitation of changes in the nucleotide pool under the various conditions and correlation of these changes with enzyme production. This is the subject of a separate investigation. The fact that both monoesterase and diesterase are induced and repressed simultaneously lends additional support to the single protein concept.

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REFERENCES

1 SHEILA A. BERRY AND J. N. CAMPBELL, *Biochim. Biophys. Acta*, 132 (1967) 78.
2 SHEILA A. BERRY AND J. N. CAMPBELL, *Biochim. Biophys. Acta*, 132 (1967) 84.
3 C. C. RICHARDSON AND A. KORNBERG, *J. Biol. Chem.*, 239 (1964) 242.
4 H. S. LORING, J. E. MCLENNAN AND T. L. WALTERS, *J. Biol. Chem.*, 241 (1966) 2876.
5 Y. ANRAKU, *J. Biol. Chem.*, 239 (1964) 3412.

6 M. S. CENTER AND F. J. BEHAL, *J. Biol. Chem.*, 243 (1968) 138.
7 J. N. CAMPBELL, J. B. EVANS, J. J. PERRY AND C. F. NIVEN, *J. Bacteriol.*, 82 (1961) 823.
8 B. N. AMES AND D. T. DUBIN, *J. Biol. Chem.*, 235 (1960) 769.
9 B. J. DAVIS, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
10 A. ALBERT, *Federation Proc.*, 20, Suppl. 10 (1961) 137.
11 SHEILA A. BERRY, Ph. D. Thesis, Department of Microbiology, University of Alberta, 1969, p. 68.
12 M. DIXON AND E. C. WEBB, *The Enzymes*, Longmans, Green and Co., London, 2nd ed., 1965, p. 84.
13 L. M. STURGE AND V. P. WHITTAKER, *Biochem. J.*, 47 (1950) 518.
14 D. K. MYERS, *Biochem. J.*, 51 (1952) 303.