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by Entamoeba histolytica

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CARBON DIOXIDE ASSIMILATION BY *ENTAMOEBA HISTOLYTICA*

By

Marlyn Ann Hamborg

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ABSTRACT

CARBON DIOXIDE ASSIMILATION BY
ENTAMOEBA HISTOLYTICA

By

Marlyn Ann Hamborg

Entamoeba histolytica incorporated ^{14}C- sodium bicarbonate into the cold trichloroacetic acid (TCA) soluble, the ethanol ether soluble, the hot TCA soluble, and the hot TCA insoluble fractions. Two dimensional thin layer chromotgraphy (TLC) of the formic acid hydrolysate of the hot TCA soluble fraction showed that the isotope was incorporated into adenine, guanine, cytosine and uracil, but not thymine. The failure of detecting thymine was probably due to the low concentration of thymine in the cell. Three unidentified substances were located on the thin layer plates. Only one of these spots (unknown #1) had an absorbtion maxima between 240-350 nm.

Entamoeba showed stimulated growth with the addition of 1.25 umoles of the nitrogenous bases, nucleosides, or nucleotides in the presence of 10 percent CO_2. Adenosine-5'-phosphate (AMP), improved growth the best

in the absence of CO_2 over the control, followed by adenine, guanosine-5'-phosphate (GMP), cytidine-5'-phosphate (CMP), and uridine-5'-phosphate (UMP).

To Bill

ACKNOWLEDGMENTS

This research was made possible through the generous support of Dr. R. Neal Band. His cooperation, guidance, and encouragement were necessary for the completion of this thesis project. Thank you, Dr. Band, for your continual support.

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ABBREVIATIONS

Abbreviations used are: TLC, thin layer chromotography; TCA, trichloracetic acid; R-5-P, ribose-5'-phosphate; AMP, adenosine-5'-phosphate; GMP, guanosine-5'-phosphate; UMP, uridine-5'-phosphate; CMP, cytidine-5'-phosphate.

INTRODUCTION

Carbon dioxide is a common requirement for gut dwelling microorganisms (Dehority, 1971; Huhtanen, Carleton and Roberts, 1954), which is also the case for E. histolytica (Band and Cirrito, 1979). In bacteria, yeast extract can spare this requirement (Lwoff and Monod, 1947). In Entamoeba, although the addition of yeast extract to a culture improves the growth it does not replace the CO_2 requirement (Band and Cirrito, 1979).

Reeves and West (1980) reported that adenosine was effective in stimulating the growth of Entamoeba when cultivated in Diamond's TP-S-1 medium which was made flavin deficient. These authors also noted that the four of the five 5'-mononucleotides also produced very good growth.

Nakamura and Baker (1956) tested a large variety of compounds for their ability to improve axenic growth of Entamoeba histolytica, and found that the compounds involved in nucleic acid metabolism gave a good response. The combination of all five nitrogenous bases gave the best response, while good growth was also seen with them individually. Methylthioadenosine was also found to be

a potent growth factor (Nakamura, 1957). Many purine and pyrimidine analogs inhibited growth and multiplication of E. histolytica, especially 2-aminopyrimidine, 2 dichloroacetamidopyrimidine, 4-dichloroacetamido-2, 6-dimethypyrimidine and 8-azaquanine. With some of the analogs the inhibition could be reversed by the addition of certain purines or pyrimidines (Nakamura and Jonsson, 1957).

Wittner (1968) developed a semi-defined medium for the axenic growth of Entamoeba which included the five nitrogenous bases as essential ingredients.

The rate of incorporation of purines, pyrimidines, and their derivatives by Entamoeba histolytica varies. Adenine and adenosine are taken up to the highest degree, followed by guanosine and guanine. All three nucleosides are incorporated more easily than the respective free base. The amebae preferred cytidine over uridine, and least thymidine (Booden, Boonlayangoor and Albach, 1976).

It is known that Entamoeba takes up adenine, adenosine, and guanosine, in part, by a "carrier"-mediated system, and guanine, hypoxanthine, and inosine by diffusion. There is also some evidence to indicate that additional transport sites for adenine, adenosine, and guanosine are present (Boonlayangoor, Albach, Stern and Booden, 1978).

It was the objective of this study to determine the importance of CO_2 to the growth of this ameba, and the fate of CO_2 incorporation during growth. Particular attention was directed to incorporation of CO_2 into purines and pyrimidines. It is known that CO_2 is used as a carbon source for the C_2 of pyrimidines, and the C_6 of purines (Lehniger, 1975). ^{14}C-sodium bicarbonate was added to growing cultures of Entamoeba, which were then fractionated by TCA precipitation. The hot trichloroacetic acid soluble extract was separated by two dimensional thin layer chromotography, and the purines and pyrimidines were examined for incorporation of the isotope.

The five basic nitrogenous bases, selected nucleosides and nucleotides were added exogenously to unmodified Diamond's TP-S-1 medium to study their ability to spare CO_2.

MATERIALS AND METHODS

^{14}C-Sodium Bicarbonate Uptake

Entamoeba histolytica, strain H-K9, was cultured axenically by the method of Band and Cirrito (1979). The cells were incubated in sealed 200 x 25 mm test-tubes (Kimax) in the modified TP-S-1 medium for 72 hrs at 35.5°C. All test-tubes were initially inoculated with 2×10^4 organisms/ml. The amebae were chilled for 5 min in an ice-water bath and harvested by centrifugation in an International centrifuge, universal model, at 300 x g for ten min at 25°C. The cell pellets were pooled to achieve a total count of 7×10^7 cells and then were washed twice with 50 ml of buffered saline, pH 7.0 (Serrano and Reeves, 1975). The composition of the buffer was 0.1 M NaCl, 20mM k_2HPO_4, 0.5 mM $MgCl_2$ and 0.1mM $Ca(NO_3)_2$. The final pellet of amebae, approximately 1 ml, was resuspended in 9.5 ml of complete TP-S-1 medium, and transferred to a 50 ml Micro-Fernbach flask (Bellco Glass Inc., New Jersey) which was sealed with a modified rubber stopper (Band and Cirrito, 1979).

The flask was flushed for 2 mins with a mixture consisting of 10 percent CO_2 in argon. This particular

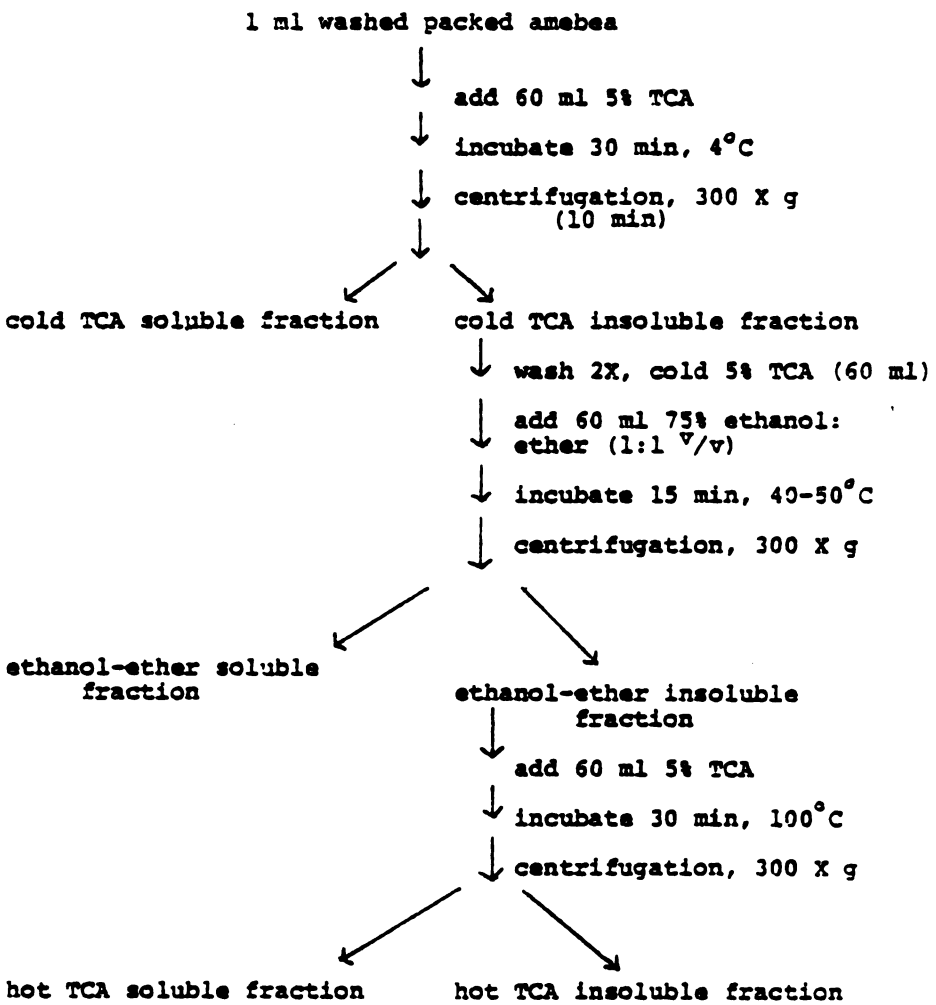
gas mixture provided optimum growth conditions (Band and Cirrito, 1979). The amebae were allowed to adjust to growing conditions in a 35.5°C water-bath for 15 mins before addition of the ^{14}C-isotope.

A 500 uCi aliquot of ^{14}C-sodium bicarbonate ($10^{-5}M$) with a specific activity of 52 mCi/m mole (New England Nuclear) was added through the inlet valve of the rubber stopper. The cells were pulse-labeled for 5, 15, and 30 mins, with occasional shaking. At the end of labeling the flasks were immediately submerged into an ice-water bath, and gassed with 100 percent argon for 2 mins. The gas leaving the flask was directly passed through two 15 ml aliquots of 0.25N NaOH in series to trap any radioactive carbon dioxide that was in the gas phase. Entamoeba was harvested by centrifugation for 10 mins at 300 x g at 25°C in an International clinical centrifuge. The pellet of cells was washed twice with buffer, pH 7.0 before TCA fractionation.

TCA Fractionation Procedure

The TCA fractionation procedure can be seen in the flow diagram below (Roberts, Abelson, Cowie, Bolton, and Britten, 1955). One ml aliquots of each step throughout the TCA fractionation were placed in scintillation vials containing 10 ml of Aquasol (New England Nuclear) to determine the amount of ^{14}C-sodium bicarbonate

TCA Fractionation Procedure



incorporated. The hot TCA soluble fraction was the main point of interest in this study.

Nucleic Acid Hydrolysis

Before hydrolysis TCA was removed from the hot TCA soluble fraction by extraction with ether (1:1 $^{v}/v$), 4 times. An absorbance reading on a Gilford spectrophotometer, model 2400-2, was taken at 260 nm to determine the amount of nucleic acid present (Warburg and Christian, 1942). This was necessary in order to know the concentration of nucleic acid added to TLC plates. 5 ml aliquots of the extracted hot TCA soluble fraction were evaporated to dryness under reduced pressure at 25°C. The residue was then dissolved in 0.25 ml of 88 percent formic acid (Fisher Scientific Co., New Jersey) and vacuum sealed according to the methods described by Wyatt and Cohen (1953). After hydrolysis, at 175°C, for 30 mins the sample was evaporated to dryness under reduced pressure at 25°C. The residue was redissolved in 100 ul of 1N HCl.

TLC of the Nitrogenous Bases of E. histolytica

The plates were prerun in the first solvent of isopropanol:HCl:H_2O (65:16.7:18.3, $^{v}/v$) to improve separation of the spots (Grippo, Iccarion, Rossi and Scarano, 1965), and then spotted with a 25 ul (0.15 mg)

aliquot of the hydrolyzed hot TCA soluble fraction dissolved in 1N HCl. The plates were developed at room temperature until the solvent front reach 13 cm, and dried overnight at 20°C or 10 min in a ventilated oven at 40°C.

N-butanol:H_2O (86:14, v/v) (Grippo, Iaccarion, Rossi and Scarano, 1965) was the solvent used to develop the plates in the second direction. The solvent was allowed to migrate for 11 cm at 25°C.

The pure nitrogenous bases, for standardization, uracil, cytosine, and guanine were obtained from California Biochemical Research; and thymine, adenine, xanthine, 1-methyl adenine, and inosine were purchased from Sigma.

The position of the various nitrogenous bases on the TLC plates was determined by viewing with ultraviolet illumination.

Liquid Scintillation Counting

Each nitrogenous base was scraped from TLC plates, and directly placed in a scintillation vial, or dissolved first in 0.1N KOH or 0.1N HCl and then counted. Either method gave comparable results. A 25 ul aliquot (0.15 mg) of the hydrolyzed hot TCA soluble residue was placed in a vial for counting.

Scintillation counting was performed in a Packard liquid scintillation spectrometer, model 3320.

Quench corrections for each sample were determined by using the external standard method (Kobayash and Maudsley, 1974). Counting efficiency for the various samples ranged from 22 to 55 percent.

Spectrophotometry of Nitrogenous Bases

Each nitrogenous base scraped from the Avicel cellulose plate was dissolved in 1 ml of 0.1N KOH or 0.1 N HCl, and the solution was centrifuged for 5 min to remove the Avicel cellulose particles. The samples were then scanned continuously between the wavelengths of 240 and 350 nm by using a Gilford spectrophotometer, model 2400-2.

Purified nitrogenous bases were also chromotographed on Avicel cellulose TLC plates, scraped and dissolved in 0.1N KOH or 0.1N HCl, to obtain an absorbance reading of known samples.

To determine the concentration of the hydrolyzed, hot TCA soluble residue a 25 ul aliquot was dissolved in 1 ml of 0.1N HCL and its absorbance read at 260 nm.

Growth Experiments

Amebae, 2×10^4, were added to 10 ml of complete TP-S-1 medium with or without the addition of nucleotides, nucleosides or nitrogen bases.

The nitrogenous bases: adenine, guanine, cytosine, uracil, and thymine; the nucleosides: adenosine and guanosine: and the nucleotides: adenosine-5'-phosphate (AMP), guanosine-5'-phosphate (GMP), cytidine-5'-phosphate (CMP), and uridine-5'-phosphate (UMP) were made up separately at a concentration of 83 uM. These were sterilized by filtration (0.45 um membrane filter, Millipor Corp.), and 0.15 ml of each compound was added to the complete TP-S-1 medium for a final concentration of 1.25 umoles. It was necessary to dissolve guanine in a small volume of 1N HCl initially, before adjusting to the final concentration of 83 uM. The small volume of 1N HCl did not affect the growth of the amebae. Guanosine, AMP, and CMP were purchased from California Biochemical Research, and UMP, and GMP were bought from Sigma.

Trophozoites in the complete medium with or without the added nucleosides, nucleotides, or nitrogenous bases were transferred to 50 ml Micro-Fernback flasks which were sealed with the modified rubber stopper.

The flasks were then gassed with 100 percent argon or a mixture of 10 percent CO_2 and 90 percent argon for 2 min, twice daily.

The cells were harvested after 72 hr at 35.5^{o}C and counted with the aid of a hemocytometer.

Statistics

Means and standard deviations were calculated on the CO_2 sparing experiments, Rf valves, and the dpm of the TCA fractions, and chromatographed nitrogeneous bases.

Two way Analysis of Variance of the CO_2 sparing experiments was also calculated. This was done on a Hewlett Packard calculator, model 9825A. The Bone-ferroni t-test was computed for the CO_2-sparing experiments in order to determine whether the addition of the nucleotides, nucleosides, or nitrogenous bases had a stimulatory effect on the growth of the amebae over the control. This was analyzed according to the method presented in Gill (1979).

RESULTS

Sodium Bicarbonate Uptake in in E. histolytica

The incorporation of ^{14}C-sodium bicarbonate (500 uCi) was studied at 5, 15, and 30 min. With experimentation it was found that in order to detect the level of radioactivity taken up by the cells into the nitrogenous bases, with the methods described in Materials and Methods, it was necessary to pulse for at least 5 min with a concentration of 500 uCi (0.9 mg/ 10 ml) of bicarbonate. The amebae looked healthy and attached to the surface of the flask at the termination of each experiment.

The incorporation of the isotope into the amebae was studied by using TCA fractionation of cells. Table 1 expresses the distribution of the isotope into the four cell fractions (cold TCA soluble, ethanol-ether soluble, hot TCA soluble, and hot TCA insoluble) with a 5 min pulse of ^{14}C-sodium bicarbonate. The highest incorporation was $\bar{x}$ = 62.5 percent, which was located in the cold TCA soluble fraction. Because this fraction contains a multitude of small molecules, it is not surprising with such a short pulse that the incorporation of the isotope was concentrated here. This fraction also had the

TABLE 1.--^{14}C-Sodium Bicarbonate Uptake into Growing Cells of *E. histolytica* in a 5 Min Pulse.

TCA Fraction	Exp 1		Exp 2		Mean ± S.D.	Mean %
	Total Counts*	%	Total Counts*	%		
Cold TCA soluble	11.6×10^6	61	5.7×10^6	64	$8.7 \times 10^6 \pm 4.2 \times 10^6$	62.5
Ethanol-ether soluble	1.8×10^6	9	0.7×10^6	8	$1.3 \times 10^6 \pm 7.4 \times 10^5$	8.5
Hot TCA soluble	5.5×10^6	29	2.4×10^6	27	$4.0 \times 10^6 \pm 2.2 \times 10^6$	28.0
Hot TCA insoluble	0.2×10^6	1	0.1×10^6	1	$0.2 \times 10^6 \pm 8.8 \times 10^4$	1.0
Totals of above	19.1×10^6	100	8.9×10^6	100		100

*These values are an average of 3 determinations of dpm/min.

highest level of activity in the 15 and 30 min pulses (Tables 2 and 3).

Of the other three fractions, the hot TCA soluble fraction had the next greatest level of incorporation ($\bar{x}$ = 28 percent). This fraction is specific for the DNA and RNA molecules, and a small amount of protein (Roberts, Abelson, Cowie, Bolton, and Britten, 1955). Because of this fraction's specificity and its level of incorporation of the isotope, this author turned her attention to the incorporation of the isotope into it.

The ethanol-ether soluble fraction, which extracts phospholipids and lipoproteins from the cell (Roberts, Abelson, Cowie, Bolton, and Britten, 1955), had an average uptake of 1.3×10^6 dpm/min. Finally, the hot TCA insoluble residue, with an incorporation of 1 percent, had the lowest uptake of ^{14}C-sodium bicarbonate. This residue contains proteins, peptides, and peptide fragments (Roberts, Abelson, Cowie, Bolton, and Britten, 1955).

The incorporation of the ^{14}C-sodium bicarbonate into the various fractions showed a slightly different distribution with a 15 min incubation. The level of incorporation into the cold TCA soluble fraction has dropped from $\bar{x}$ = 64 percent to $\bar{x}$ = 56.3 percent, and an increase can be seen in the ethanol-ether soluble, hot TCA soluble, and hot TCA insoluble fractions (Table 2).

TABLE 2.--^{14}C-Sodium Bicarbonate Uptake into Growing Cells of *E. histolytica* in a 15 Min Pulse.

TCA Fraction	Exp 5*	
	Total Counts	%
Cold TCA soluble	4.9×10^6	56.3
Ethanol-ether soluble	1.1×10^6	12.2
Hot TCA soluble	2.4×10^6	28.0
Hot TCA insoluble	0.3×10^6	3.5
TOTALS of above	8.1×10^6	100.0

*These values are an average of 3 determinations of dpm/min.

TABLE 3.--^{14}C-Sodium Bicarbonate Uptake Into Growing Cells of E. histolytica in a 30 Min Pulse.

TCA Fraction	Exp 3		Exp 4		Mean ± S.D.	Mean %
	Total Counts*	%	Total Counts*	%		
Cold TCA soluble	2.4×10^6	35.3	4.6×10^6	47.9	$4.0 \times 10^6 \pm 8.6 \times 10^5$	41.7
Ethanol-ether soluble	1.6×10^6	17.0	0.5×10^6	5.3	$1.1 \times 10^6 \pm 7.9 \times 10^5$	11.1
Hot TCA soluble	4.4×10^6	46.1	4.0×10^6	41.5	$4.2 \times 10^6 \pm 3.1 \times 10^5$	43.8
Hot TCA unsoluble	0.1×10^6	1.6	0.5×10^6	5.3	$0.3 \times 10^6 \pm 2.5 \times 10^5$	3.4
Totals of above	9.6×10^6	100.0	9.6×10^6	100.0		100.0

*These values are an average of 3 determinations of dpm/min.

Table 3 presents the data of a 30 min pulse with 500 uCi of ^{14}C-sodium bicarbonate. The incorporation into the cold TCA soluble fraction has continued to drop. It appears that during this 30 min time span some of the smaller precursor molecules in the cold TCA soluble fraction are being biosynthesized into their larger products. However, one must keep in mind that 30 mins is a very short period in this organism's generation time (t = 10 hrs). The level of uptake into the ethanol-ether soluble fraction and the hot TCA insoluble fraction showed an increase up to 15 min, but have stabilized between the 15 and 30 min time period. Even though the ethanol-ether fraction of one experiment with a 30 min pulse has a lower level of incorporation of the isotope (5.3 percent) than either the 5 or 15 min pulse experiments, the combined evidence of all the other experiments shows a trend of gradual increase of the isotope over time. This discrepancy could be attributed to the fact that each experiment was done with different cultures of cells (Monad, 1949). Also the total recovery of the isotope was low and variable with each experiment (33 to 78 percent). Experiment #1 had a 78 percent recovery of the isotope which is probably the reason for such a high total count (19.1×10^6). All four other experiments had a recovery rate no higher than 45 percent.

However, the level of incorporation into the hot TCA soluble fraction has increased from $\bar{x}$ = 28 percent at 5 and 15 min to $\bar{x}$ = 43.8 percent at 30 min. Only one experiment was done with the 15 min incubation of isotope, so it is not possible at this time to say whether any increase of the isotope occurred between 5 and 15 min, but a definite increase can be seen between 5 and 30 mins.

Thin Layer Chromotography and Ultraviolet Absorbance Spectra of E. histolytica's Nitrogenous Bases

Two dimensional ascending chromotography was used to separate the various nitrogenous bases in order to determine the level of radioactivity in each compound (spot). The ^{14}C-sodium bicarbonate uptake into the nitrogenous bases is discussed later in the results section. The spot location of each nitrogenous base was determined by viewing under ultraviolet light (Figure 1), and Rf values calculated normally. The Rf values for each experiment and control can be found in Tables 4, 5, 6 and 7. The migration of E. histolytica's nitrogenous bases is very comparable to the migration of pure bases bought commercially. The small variation in the Rf values of the many plates developed did not cause confusion due to the fact that the spots always maintain the same chromatographic pattern. The slight differences

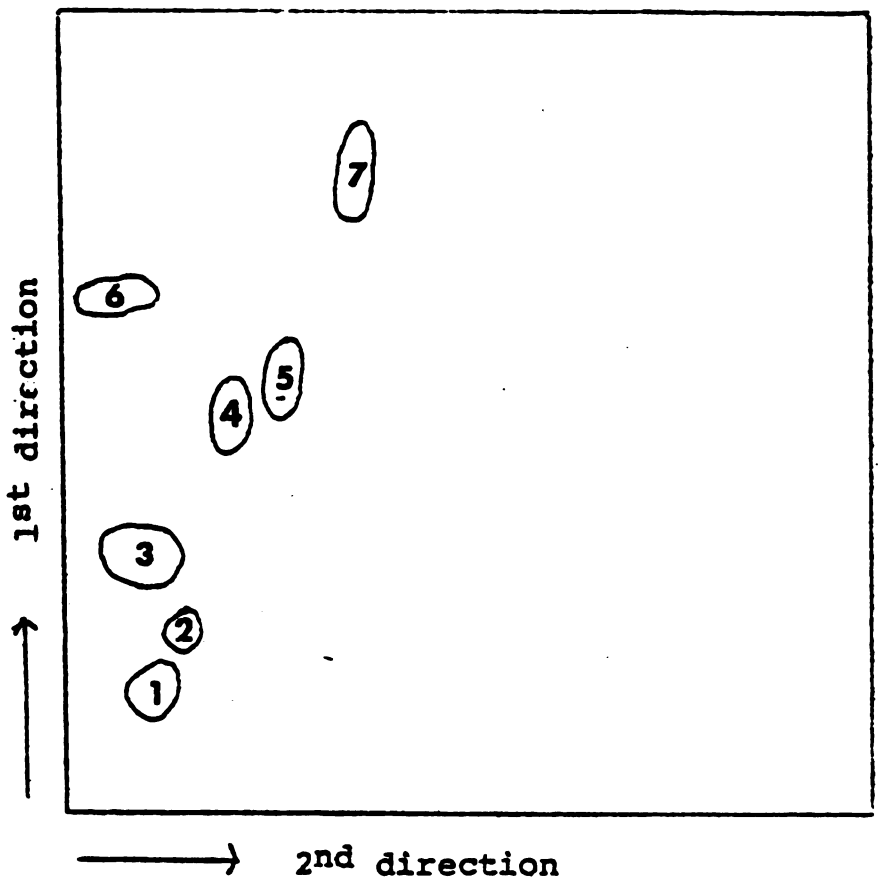


Figure 1.--Two dimensional thin layer chromography of E. histolytica's nitrogenous bases. (1 - adenine, 2 - guanine, 3 - cytosine, 4 - unknown #1, 5 - uracil, 6 - unknown #2, 7 - unknown #3.)

TABLE 4.--Rf Values of Pure Nitrogenous Bases Developed in Isopropanol:HCl: H_2O (65:16.7:18.3, v/v).

Nitrogenous Base	Exp 1	Exp 2	Exp 3	Mean ± S.D.
Adenine	.29	.32	.32	.31 ± .02
Cytosine	.43	.38	.35	.39 ± .04
Guanine	.17	.10	.19	.15 ± .05
Uracil	.60	.68	.62	.63 ± .04
Inosine	.40	.38	.39	.39 ± .01
1-methyl adenine	.47	.47	.45	.46 ± .01

TABLE 5.--Rf Values of E. histolytica's Nitrogenous Bases Developed in Isopropanol:HCl:H_2O (65:16.7:18.3, v/v).

Nitrogenous Bases	Exp 1*		Exp 2*		Exp 3**		Exp 4**		Exp 5***		Mean ± S.D.
	A†	B†	A†	B†	A†	B†	A†	B†	A†	B†	
Adenine	.35	.30	.25	.24	.17	.25	.27	.30	.21	.21	.25 ± .05
Cytosine	.58	.38	.43	.36	.35	.33	.39	.41	.30	.30	.38 ± .08
Guanine	.15	.16	.16	.10	.08	.10	.15	.16	.09	.10	.12 ± .03
Uracil	.59	.65	.61	.54	.53	.58	.62	.62	.51	.54	.58 ± .05
Unknown #1	.53	.64	.60	.53	.50	.57	.60	.60	.50	.51	.56 ± .05
Unknown #2	.68	.69	.68	.62	.75	.75	.60	.70	.66	.66	.68 ± .05
Unknown #3	.86	.83	.73	.84	.76	.62	.71	.87	.74	.73	.76 ± .08

*Exp 1 and Exp 2 are from a 5 min pulse of ^{14}C-sodium bicarbonate.

**Exp 3 and Exp 4 are from a 30 min pulse of ^{14}C-sodium bicarbonate.

***Exp 5 is a 15 min pulse of ^{14}C-sodium bicarbonate.

†Exps A and B are two different chromatographic separations.

TABLE 6.--Rf Values of Pure Nitrogenous Bases Developed in n-Butanol:H_2O (86:14, v/v).

Nitrogenous Bases	Exp 1	Exp 2	Exp 3	Mean ± S.D.
Adenine	.13	.15	.17	.15 ± .02
Cytosine	.12	.17	.11	.13 ± .03
Guanine	.08	.07	.04	.06 ± .02
Thymine	.53	.54	.45	.51 ± .05
Uracil	.37	.37	.32	.35 ± .03
Inosine	.11	.19	.16	.15 ± .04
1-methyl adenine	.47	.48	.48	.48 ± .006

TABLE 7.--Rf Values of E. histolytica's Nitrogenous Bases Developed in n-Butanol: H_2O, (86:14, v/v).

Nitrogenous Bases	Exp 1*		Exp 2*		Exp**		Exp 4**		Exp 5***		Mean ± S.D.
	A†	B†	A†	B†	A†	B†	A†	B†	A†	B†	
Adenine	.15	.11	.19	.20	.10	.17	.16	.17	.18	.16	.16 ± .03
Cytosine	.04	.04	.07	.06	.09	.05	.08	.07	.10	.07	.07 ± .02
Guanine	.08	.02	.04	.10	.07	.13	.05	.09	.14	.08	.08 ± .04
Uracil	.26	.21	.38	.38	.26	.36	.35	.35	.41	.27	.32 ± .01
Unknown #1	.14	.12	.27	.25	.16	.14	.26	.24	.29	.15	.20 ± .06
Unknown #2	doesn't migrate										
Unknown #3	.52	.52	.53	.55	.32	.57	.45	.39	.41	.40	.46 ± .08

*Exps 1 and 2 are the 5 min pulse of ^{14}C-sodium bicarbonate.

**Exps 3 and 4 are the 30 min pulse of ^{14}C-sodium bicarbonate.

***Exp 5 is the 15 min pulse of ^{14}C-sodium bicarbonate.

†Exps A and B are two different chromatographic separations.

in the Rf values could be attributed to small changes in temperature or different batches of thin layer plates.

Parallel migration in Isopropanol:HCl:H_2O (65:16.7:18.3 $^{v}/v$) of spots believed to be adenine, cytosine, guanine, and uracil were compared against purified samples, and the results can be seen in Table 8. A spectrophotometric analysis of each spot was also done.

Spots from E. histolytica's hot TCA soluble fraction believed to be adenine, cytosine, guanine, and uracil were also identified by determining the absorbance maxima of each between the wavelengths of 240 and 350 nm in 0.1N HCl, and 0.1N KOH (Table 9 and Figures 2-5). The combination of two dimensional chromotographic separation and spectrophotometric analysis gives strong evidence that the spots believed to be adenine, cytosine, guanine, and uracil are indeed so.

Two spots separated by TLC were seen while viewing under the ultraviolet light which did not correspond to any of the known nitrogenous bases (uracil, cytosine, thymine, guanine, and adenine), minor bases (1 methyl-adenine) or precursors (xanthine and inosine) checked. One of the spots was unknown #1 which had Rf values of $\bar{x}$ = .56 and $\bar{x}$ = .20 in the first and second direction of development, respectively. This particular

TABLE 8.--Rf Values of Parallel Migration of E. histolytica's Nitrogenous Bases and Purified Samples in Isopropanol:HCl:H_2O,(65:16.7:18.3, $^{V}/v$).

Nitrogenous Base	E. histolytica's	Purified
Adenine	.27	.27
Cytosine	.35	.35
Guanine	.16	.16
Uracil	.58	.58

TABLE 9.--Absorbance Maxima of Nitrogenous Bases.

Compound	.1N HCl nm	.1N KOH nm
Adenine	274	269
Guanine	249	275
Cytosine	278	282
Uracil	258	284
Thymine	265	291
Inosine	260	254
Xanthine	261	247
1-methyl adenine	259	271
Unknown #1	261	262

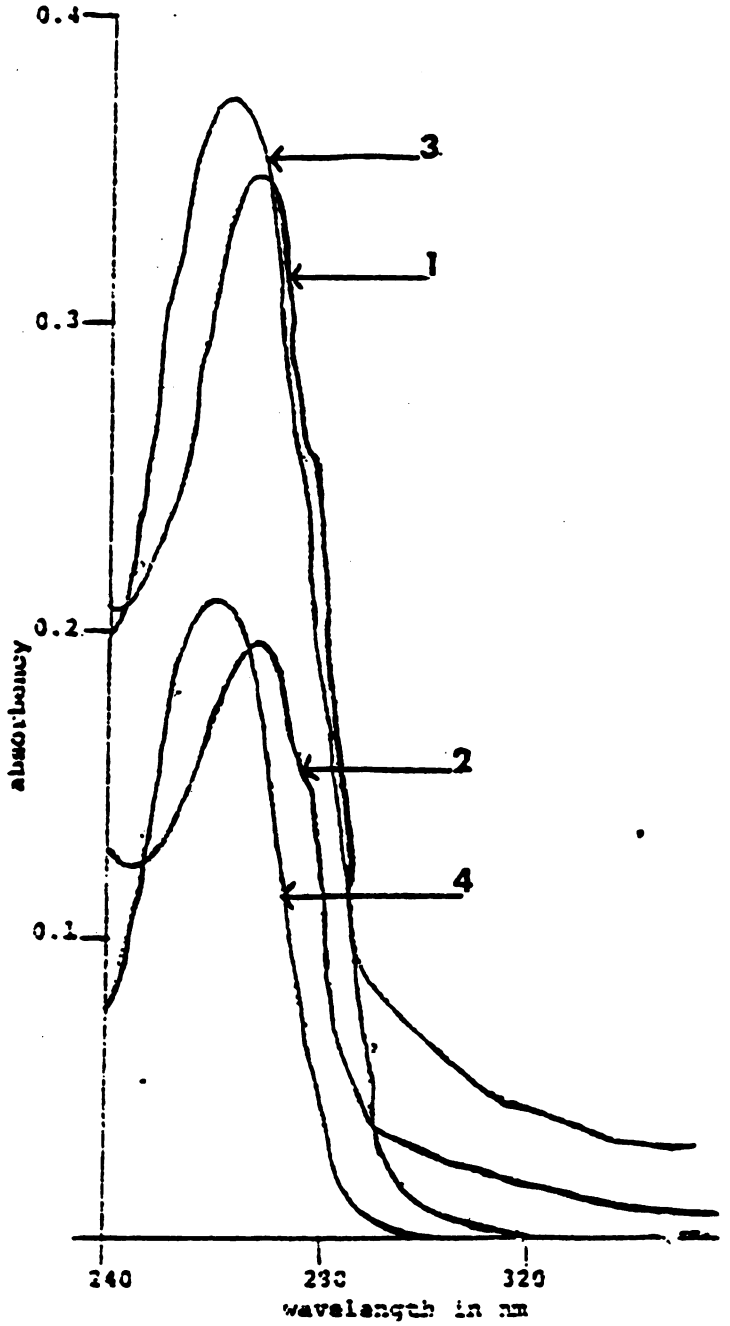


Figure 2.--Ultraviolet absorption spectra of E. histolytica's adenine. (1 and 2 are the pure sample's and Entamoeba's base in 0.1N KOH, and 3 and 4 are the pure samples and Entamoeba's base in 0.1N HCl.

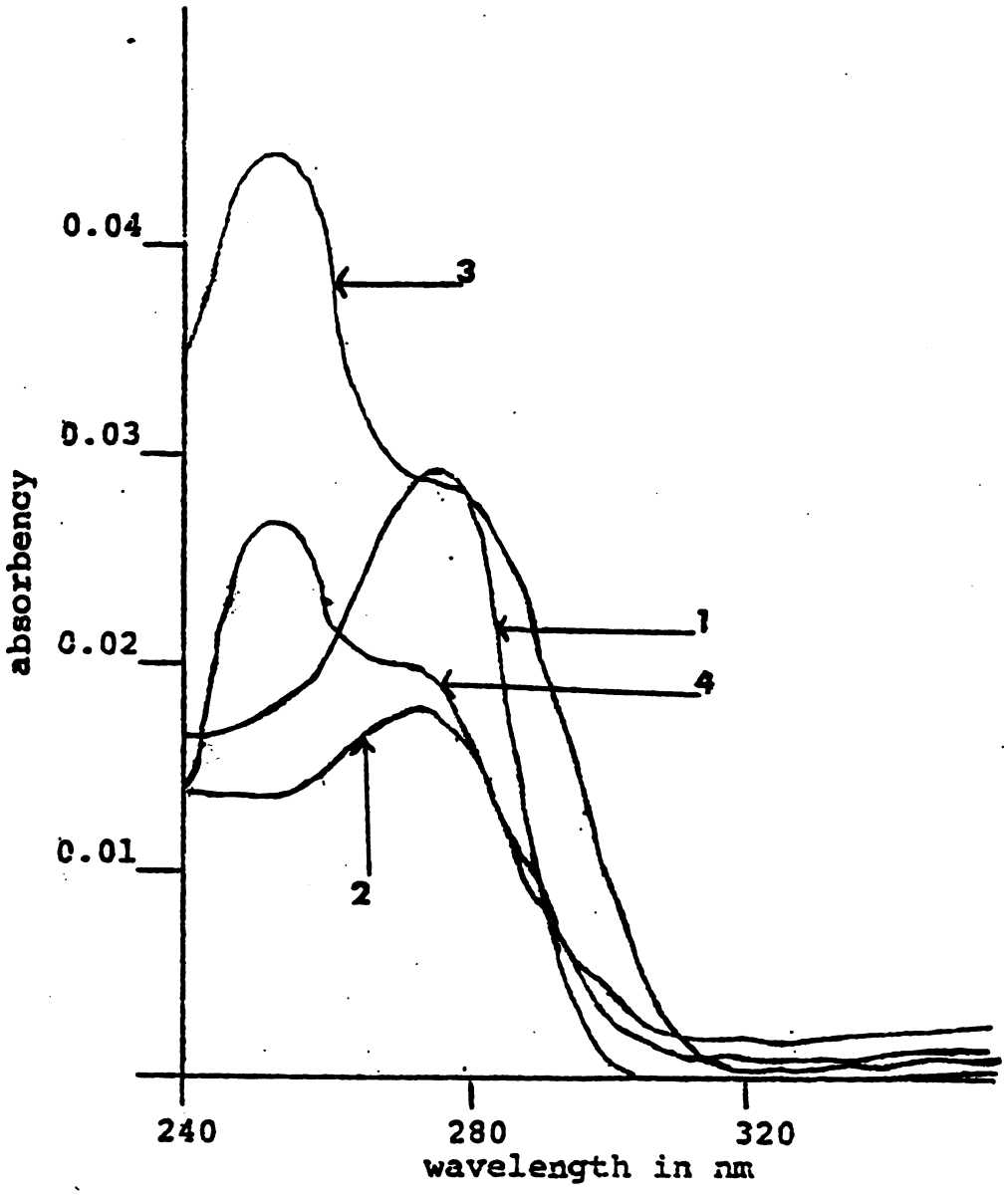


Figure 3.--Ultraviolet absorption spectra of E. histolytica's guanine. (1 and 2 are the pure sample's and Entamoeba's base in 0.1N KOH, and 3 and 4 are the pure sample's and Entamoeba's base in 0.1N HCl.)

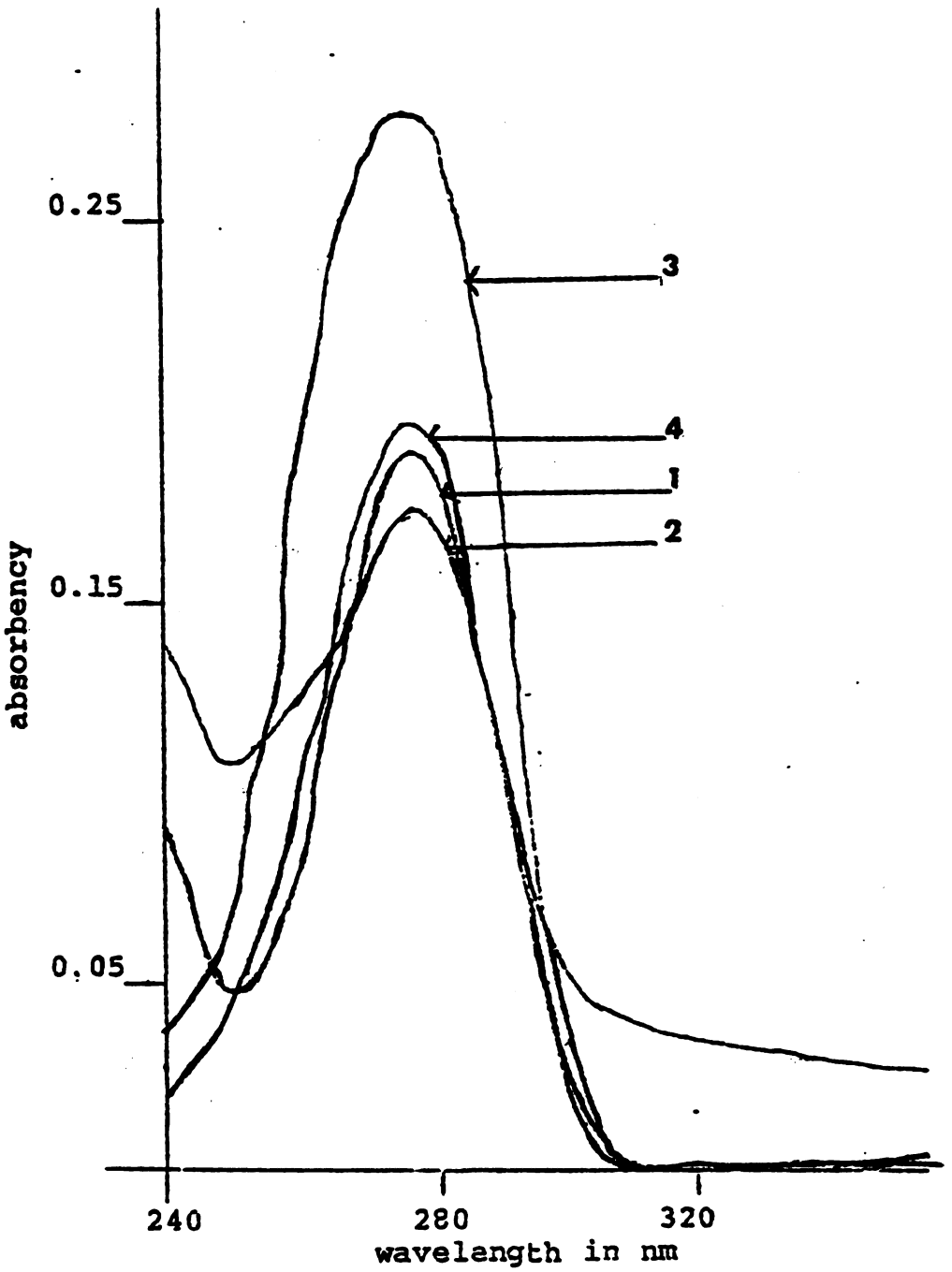


Figure 4.--Ultraviolet absorption spectra of E. histolytica's cytosine. (1 and 2 are the pure sample's and Entamoeba's base in 0.1N KOH, and 3 and 4 are the pure sample's and Entamoeba's base in 0.1N HCl.)

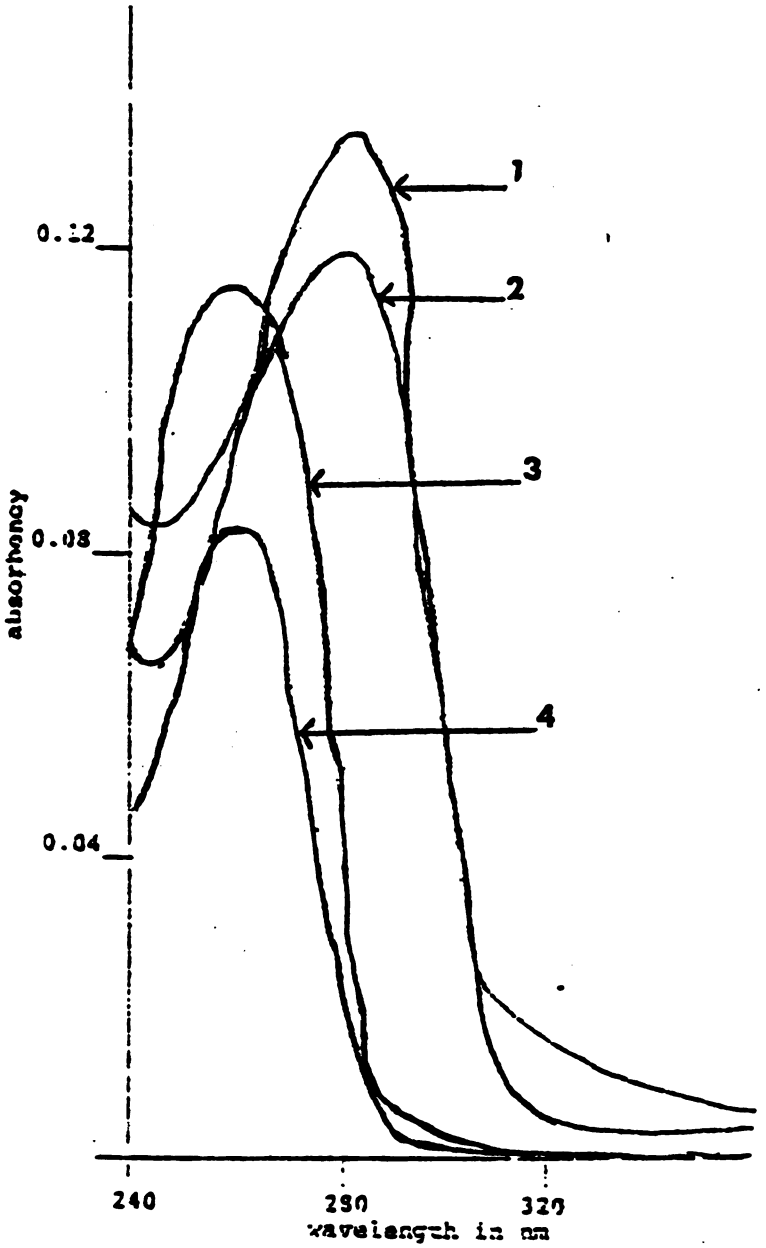


Figure 5.--Ultraviolet absorption spectra of E. histolytica's uracil. (1 and 2 are the pure sample's and Entamoeba's base in 0.1N KOH, and 3 and 4 are the pure sample's and Entamoeba's base in 0.1N HCl.)

spot has an absorbance maxima of 262 nm in 0.1N KOH and 261 nm in 0.1N HCl (Figure 6). This spot has been checked against such knowns as xathine, thymine, inosine, and 1-methyl adenine, but the absorbance spectra and Rf values do not coincide.

Unknown #2, the second spot, absorbed ultraviolet light strongly, but did not give an absorbance maxima between 240 nm and 350 nm. Its Rf value in the first solvent was $\bar{x}$ = .68, and it did not migrate in the second solvent of TLC.

A third spot, unknown #3, was removed from the general vicinity of thymine with an Rf value of $\bar{x}$ = .76 in the first direction of development, and $\bar{x}$ = .46 in butanol - HCl. This spot, also, did not produce any discernible peak between 240 and 350 nm. The amount of thymine in the cell is very small and difficult to recover (Gelderman, Bartgis, Keister, and Diamond, 1971); therefore, the concentration may have been too low for detection spectrophotometrically. However, there is no evidence at this time to support the fact that the spot is thymine. Bolton et al. (1952) doing similar work with E. coli were also unable to detect thymine. The identity of these unknown spots is not suspected to be a nucleoside or nucleotide because of the severity of the hydrolysis (Wyatt and Cohen, 1953).

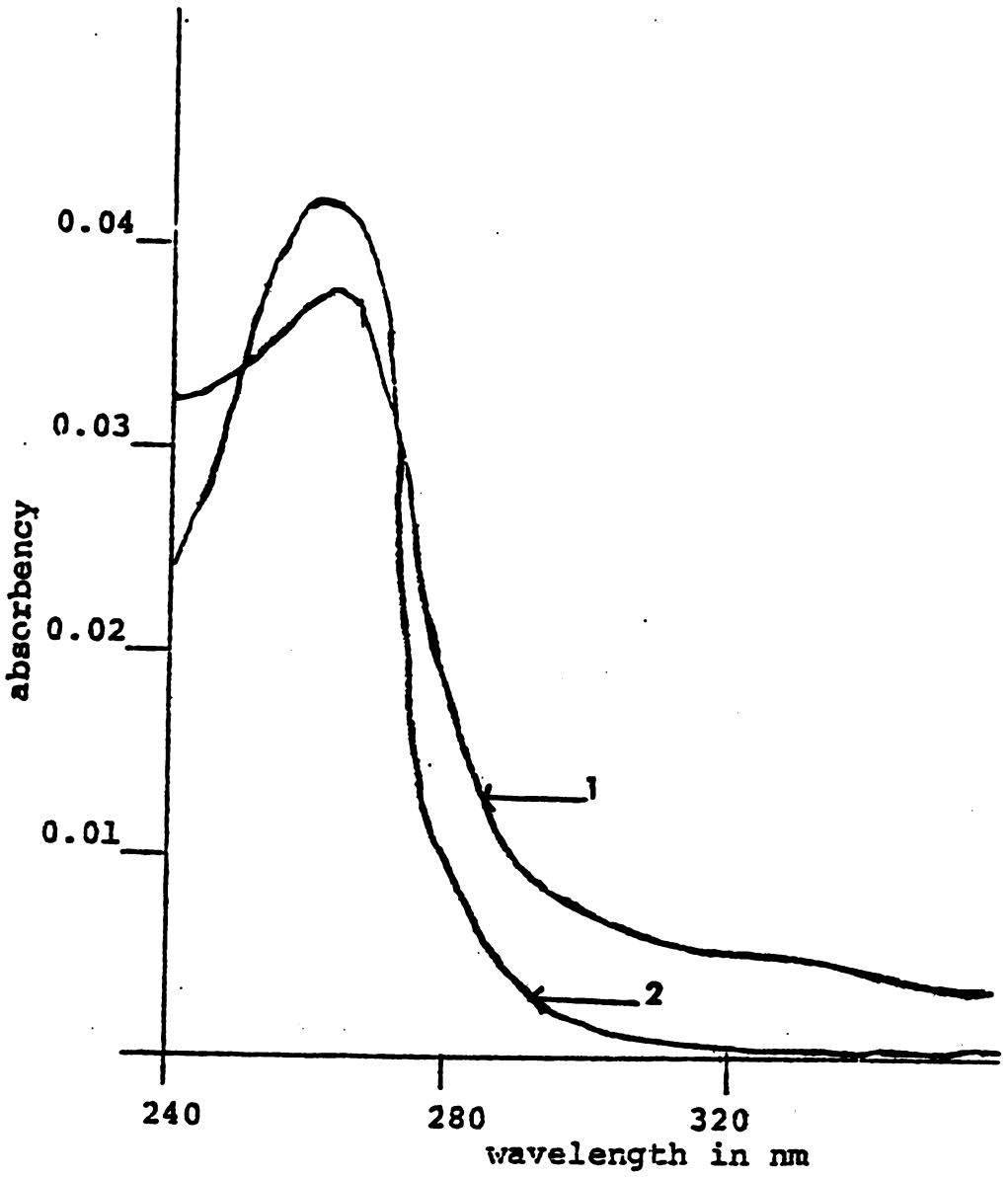


Figure 6.--Ultraviolet absorption spectra of E. histolytica's unknown #1. (1 and 2 are Entamoeba's unknown base in 0.1N KOH and 0.1N HCl, respectively.)

Absorbance of Nitrogenous Bases

An absorbance scan of all spots was completed with the 5, 15, 30 min pulses of ^{14}C-sodium bicarbonate, but only the results of one is given. All sets of absorbance spectra were comparable.

^{14}C-Sodium Bicarbonate Incorporation into E. histolytica's Nitrogenous Bases

After formic acid hydrolysis of the hot TCA soluble fraction, the nitrogenous bases and possible minor bases were separated on Avicel cellulose plates to detect the level of incorporation into each. Table 10 presents the distribution of the ^{14}C-sodium bicarbonate into the various bases when given a 5 min pulse. Of the known bases, the greatest incorporation was in cytosine ($\bar{x}$ = 277.5) and the other pyrimidine, uracil ($\bar{x}$ = 229).

The three unknown spots had an incorporation of the isotope higher than any of the known nitrogenous bases. One might suspect that these spots may be minor bases, but in high concentration in E. histolytica, because the hot TCA soluble fractions only contains polymeric molecules of DNA and RNA. The identity of these spots could not be determined at this time, so it is difficult to assess the importance or changes in the incorporation into these spots.

TABLE 10--Uptake of ^{14}C-Sodium Bicarbonate into the Nitrogenous Bases of *E. histolytica* in a 5 Min Pulse.

Nitrogenous Base	Exp 1*	Exp 2*	Mean ± S.D.
Adenine	102.1	91.2	96.6 ± 7.7
Guanine	230.9	154.2	192.5 ± 54.2
Cytosine	268.3	286.2	277.5 ± 13.1
Uracil	305.7	152.4	229.0 ± 108.4
Unknown #1	297.6	297.6	297.6 ± 0
Unknown #2	366.7	415.8	391.2 ± 34.7
Unknown #3	644.7	342.8	493.7 ± 213.5
Total spot**	39,122.9	15,868.9	27,495.9 ± 16,443.1

*These values are an average of 3 determinations of dpm/min.

**25 ul aliquot of hydrolyzed hot TCA soluble fraction (.15 mg).

By 15 min (Table 11) the incorporation of the isotope is beginning to increase in adenine, and continues to increase with a pulse of 30 min (Table 12). Incorporation into all other samples has dropped. Whether this drop in incorporation is an accurate representation of incorporation is uncertain because this experiment was performed only once.

When one looks at the distribution of the isotope into the nitrogenous bases and possible minor bases through 30 min, there is a definite increase in all spots. Of the four nitrogenous bases, adenine has the highest level of incorporation. ATP is required for many biosynthetic reactions which would correlate with a higher synthesis of adenine.

The fact that adenine has the highest level of incorporation fits nicely with the CO_2 sparing experiments presented in this paper. The results indicate that AMP allows the greatest level of growth in the absence of gaseous CO_2 over the control. Adenine being a precursor to AMP. Reeves and West (1979), using different media conditions, also found that adenosine stimulated amebae growth the best.

At this time it is not known why the 25 ul aliquot of the hydrolyzed hot TCA soluble fraction added to the TLC plates has such a large number of counts as compared to the sum of the separated spots.

TABLE 11--Uptake of ^{14}C-Sodium Bicarbonate Into the Nitrogenous Bases of E. histolytica in a 15 Min Pulse.

Nitrogenous Base	Exp 5*
Adenine	144.9
Guanine	80.0
Cytosine	137.1
Uracil	109.1
Unknown #1	181.2
Unknown #2	351.4
Unknown #3	108.3
Total spot**	23,810.0

*These values are an average of 3 determinations of dpm/min.

**25 ul aliquot of hydrolyzed hot TCA soluble fraction (0.15mg).

TABLE 12.--Uptake of ^{14}C-Sodium Bicarbonate Into the Nitrogenous Bases of *E. histolytica* in a 30 Min Pulse.

Nitrogenous Base	Exp 3*	Exp 4*	Mean ± S.D.
Adenine	533.0	98.0	315.5 ± 307.6
Guanine	366.0	80.5	223.2 ± 201.9
Cytosine	173.7	180.9	177.3 ± 5.1
Uracil	171.1	149.1	160.0 ± 15.5
Unknown #1	230.7	639.2	434.9 ± 288.8
Unknown #2	126.7	758.9	442.8 ± 447.0
Unknown #3	644.2	758.9	701.5 ± 81.1
Total spot**	25,901.4	26,686.3	26,638.3 ± 67.8

*These values are an average of 3 determinations of dpm/min.

**25 ul aliquot of hydrolyzed hot TCA soluble fraction (.15 mg).

The 1N HCl which the sample was dissolved in and the solvents used to chromatographically separate the spots did not produce a level of radioactivity above background. Also area of the plate which did not contain a spot showed no significant level of radioactivity above background. This point of interest must be pursued further.

CO_2 Sparing Experiments

The growth of E. histolytica in 50 ml Micro-Fernback flasks, which were gassed twice daily with 100 percent argon, or 90 percent argon and 10 percent CO_2 (Tables 13 and 14), in the presence of added nitrogenous bases, nucleosides, or nucleotides, supported the conclusion of Band and Cirrito (1979) that there is a significant difference in amebae growth in the presence of CO_2 in the gas phase. The statistical evidence for this can be seen in Table 15. This suggests that E. histolytica has a CO_2 requirement.

The next point of interest is the possible sparing effect of the CO_2 by nitrogenous bases, nucleosides or nucleotides. The growth response of the amebea over a three day period in the presence of the different substances can be seen in Tables 13 and 14. With the addition of each compound at a concentration of 1.25 µmole, it was found that AMP allowed the best

TABLE 13.--Growth of *E. histolytica* in the Presence of 10 Percent CO_2.

Sample	Exp A ($\times 10^6$)	Exp B ($\times 10^6$)	Exp C ($\times 10^6$)	Exp D ($\times 10^6$)	Mean ± S.D. ($\times 10^6$)
Adenine	3.3	2.7	3.2	2.8	2.9 ± 0.24
Cytosine	2.6	3.0	2.9	2.8	2.8 ± 0.17
Guanine	4.5	3.8	3.9	3.4	3.9 ± 0.45
Uracil	1.5	1.7	2.9	3.2	2.3 ± 0.85
Thymine	3.0	2.6	3.4	3.7	3.2 ± 0.49
Adenosine	2.4	2.2	3.2	2.7	2.7 ± 0.46
Guanosine	3.6	2.0	4.0	3.3	3.2 ± 0.86
AMP	3.2	2.2	2.3	2.1	2.4 ± 0.51
CMP	2.0	2.0	1.8	1.2	1.7 ± 0.38
GMP	2.8	3.0	3.2	4.1	3.3 ± 0.57
UMP	5.0	2.5	3.7	2.1	3.3 ± 1.31
All bases	4.4	0.4	4.2	3.7	4.1 ± 1.87
All NMPs	3.0	2.8	3.5	4.7	3.5 ± 1.72
None	3.2	3.6	2.4	1.6	2.7 ± 0.89

NOTES: Above values were calculated by subtracting t = 0 hr. from t = 72 hr. Above values are the number of amebae in 10 ml.

TABLE 14.--Growth of *E. histolytica* in the Absence of 10 Percent CO_2.

Sample	Exp A ($\times 10^6$)	Exp B ($\times 10^6$)	Exp C ($\times 10^6$)	Exp D ($\times 10^6$)	Mean ± S.D. ($\times 10^6$)
Adenine	0.9	3.2	1.4	1.0	1.6 ± 1.09
Cytosine	3.0	3.4	1.9	0.7	2.2 ± 1.27
Guanine	2.7	2.1	2.9	2.5	2.5 ± 0.34
Uracil	2.5	1.7	2.0	1.1	1.8 ± 0.57
Thymine	1.7	0.8	3.2	2.1	1.9 ± 1.01
Adenosine	0.1	1.8	1.4	2.5	1.4 ± 1.05
Guanine	1.8	3.5	3.1	0.7	2.3 ± 1.28
AMP	3.3	2.1	2.4	2.1	2.5 ± 0.57
CMP	1.8	2.1	0.9	1.6	1.6 ± 0.52
GMP	3.3	3.6	3.0	3.2	3.3 ± 0.25
UMP	2.4	3.1	3.7	2.1	2.8 ± 0.72
All bases	2.5	2.4	1.9	3.9	2.7 ± 0.32
All NMPs	3.4	2.5	2.8	2.5	2.8 ± 0.42
None	1.3	1.7	0.4	2.7	1.5 x 0.96

NOTES: Above values were calculated by subtracting the t = 0 hr. from t = 72 hr. Above values are the number of amebae in 10 ml.

TABLE 15.--Two Way Analysis of Variance of CO_2 Sparing Experiments.

Source	D.F.	Mean Square
Treatment	1	1.26×10^{13}
Interaction	13	5.54×10^{11}
Error	84	7.68×10^{11}
F ratio	1	84

replacement of CO_2 over the control (ρ = ~.86), where ρ = (1 - C.D.F.). AMP was followed by adenine (ρ = ~.80) > GMP (ρ = ~.80), > CMP (ρ = ~.76), > UMP (ρ = ~.68). Even though the Boneferroni t-statistic for the above experiments is only moderately strong, there is some evidence that a difference between the treated and the control does exist.

Stimulation by the nucleotides might be more evident if the flavin-deficient medium employed by Reeves (1980) was used instead of the standard TP-S-1.

The other nitrogenous bases (uracil, cytosine, thymine, and guanine), nucleosides (adenosine or quanosine), or the combination of all the nucleotides did not show a significant stimulation in growth over the control.

DISCUSSION

The entire spectrum of possible nutrient requirements of E. histolytica has been studied by a number of authors (Nakamura and Baker, 1956; Nakamura, 1957; Latour and Reeves, 1965; Wittner, 1968; Boonlayangoor, Albach, Stern and Booden, 1978; Band and Cirrito, 1979; Lo and Reeves, 1979; Reeves and West, 1980). Until 1968, when L. S. Diamond developed a medium for axenic cultivation, it was difficult to evaluate the older studies (Nakamura and Baker, 1956; Nakamura, 1957; Latour and Reeves, 1965; Wittner, 1968) which dealt with the nutritional needs of Entamoeba. The reason being it was necessary to cultivate this ameba in the presence of bacteria. Therefore, whether E. histolytica obtained its nutritional needs from the bacterial metabolites or the complex media was unclear. This paper looked into carbon dioxide and its possible interaction with nitrogeneous bases and their derivatives as growth stimulating substances.

With the addition of 1.25 µmoles of any of the nitrogenous bases, nucleosides, or nucleotides to the TP-S-1 medium, this author supported the conclusion of Band and Cirrito (1979) that the presence of CO_2 in the

gas phase above the medium improved growth of Entamoeba histolytica.

Carbon dioxide is a common requirement of gut dwelling microorganisms. Dehority (1971) studied the CO_2 requirement of 32 strains of rumen bacteria. His results indicated that the major CO_2 requirement was a biosynthetic one, in which CO_2 was required for cell growth and multiplication. It should be emphasized that many species have an absolute CO_2 requirement even in complex culture media (Dehority, 1971).

For orange-colored Streptococcus bovis CO_2 is important in the synthesis of amino acids and fatty acids (Hayashi and Kitahara, 1960; Prescott and Stutts, 1957; Prescott, Ragland and Stutts, 1957). It has been found that the CO_2 requirement can be replaced by a mixture of amino acids, or a solution of some unsaturated fatty acids and oleate-containing compounds.

E. coli is another microorganism which utilizes CO_2 in a biosynthetic mode. Carbon dioxide is incorporated into the synthesis of proteins and nucleic acids. Sixty-two percent of $NaH^{14}CO_3$ was found to be incorporated into aspartic acid, glutamic acid, arginine, lysine, proline, and threonine (Abelson, Bolton and Aldous, 1952).

The importance of CO_2 in the synthesis of lipids, in E. histolytica, was not pursued in this

paper. But an incorporation of ^{14}C-sodium bicarbonate in the ethanol-ether extract reached a level of 11 percent in 30 mins. So whether Entamoeba incorporates CO_2 into malonyl-COA formation in the synthesis of long chain fatty acids (Lehninger, 1975) is not known. Because this biosynthetic reaction is common to some cell types, it would be worthwhile to investigate the presence of this reaction in Entamoeba, and any possible nutritional implications.

Incorporation of 3 percent of $NaH^{14}CO_3$ into proteins of E. histolytica was noted over a 30 min period (Table 3). This hot TCA insoluble residue was hydrolyzed with 6N HCL for 24-48 hours and separated on two dimensional chromotography according to the methods of Detterbeck and Lillevik (1971). However, the level of incorporation of the isotope into the individual amino acids was too low for detection with the methods used.

An attempt was also made to see if a sparing effect of CO_2 could be achieved by an exogenous addition of amino acids. Using 2 mg or 0.1 mg of amino acid per ml of media depending on its solubility, the initial data seemed to indicate a possible stimulatory effect on growth. Further manipulation of the concentrations will be necessary to pinpoint specific amino acids, if any, and obtain statistically stronger evidence. Because the

incorporation of ^{14}C-sodium bicarbonate into the protein fractions was so low, the possibility remains that the amino acids present in the TP-S-1 medium, supplied by panmede liver extract and trypticase, are sufficient to meet the nutritional needs of Entamoeba. The amino acids in the media may also inhibit or reduce the incorporation of the isotope into the proteins (Abelson, Bolton, and Aldous, 1952).

Wittner (1968) developed a semi-defined medium which included eighteen amino acids. The increase in cell number over time was not as great as cells grown in Diamond's TP-S-1 media, but the shapes of the growth curves were identical. This would seem to indicate that the amebae are showing a normal growth response. Perhaps small adjustments of the concentrations or chemicals in the semi-defined media would produce a better growth response.

As for the incorporation of $NaH^{14}CO_3$ into the nucleic acid fraction (hot TCA soluble) of Entamoeba is concerned, about a 28 percent uptake was seen with a 5 min and 15 min pulse, which increased to 44 percent after 30 mins (Tables 1, 2, and 3). As in E. coli (Bolton, Abelson and Aldous, 1952) the isotope was located in both purines and pyrimidines except thymine (Tables 10, 11 and 12). E. T. Bolton et al. also noted the low recovery of thymine. As in the case of E. coli

quantitative uptake analysis of this pyrimidine could not be done here either.

Geldermar et al. (1971) found it necessary to use amebae in numbers in far excess than expected in order to determine the amount of DNA per cell (4 x 10^{-13} gm) by the standard CSCl gradient procedure. The fact that DNA isolation from the cell is difficult could explain the absence or very low concentration of thymine in the hot TCA soluble fraction.

It is known in the synthesis of pyrimidines that CO_2 is incorporated into the second carbon of the pyrimidine ring via the formation of carbamoyl-phosphatic acid by carbamoyl-phosphate synthase, and into the sixth carbon of the purine ring structure via the intermidate, 5'phosphoribosyl-5 aminoimidazole-4-carboxylic acid (Lehninger, 1975). In analyzing a 0.15 mg sample of hydrolyzed hot TCA soluble over time, the highest incorporation of CO_2 is into adenine (315.5 dpm/min). In Entamoeba the incorporation of CO_2 into guanine followed adenine, cytosine being third, and uracil last. The large loss of the isotope when the 0.15 mg sample is separated into its perspective compounds by thin layer chromotography cannot be satisfactorily explained by checking the obvious places of loss (solvents, thin layer plates or chemiluminescence). Further investigation of this point is greatly needed.

During the separation of the hydrolyzed TCA soluble fraction three spots which did not correspond to any of the five basic nitrogenous bases were noticed. Unknown #3 was one which migrated on the TLC plates very similar to thymine. However, an absorbance peak between 240 and 350 nm could not be obtained. Therefore, its identity as thymine could not be confirmed.

Unknown #2, which strongly absorbed under an ultraviolet light also did not produce any discernible peak when scanned spectrophotometrically between 240-350 nm. The absorbence of this spot or unknown #3 by ultraviolet light may simply be due to contaminating material. This could explain the inability to get an ultraviolet spectrum, but not its uptake of the isotope. Analysis of these two samples by other means such as gas chromotography or mass spectrophotometry may determine their identity.

An ultraviolet absorbance peak of 261 nm in 0.1N HCl and 262 nm in 0.1N KOH was achieved for unknown #1. Because it underwent 88 percent formic acid hydrolysis one would expect it to contain the ring structure of a purine or pyrimidine with no surviving sugar or phosphate group. Obvious possibilities for the identity of this spot would be one of the minor bases, perhaps in higher concentration in this ameba. It was checked against such knowns as xanthine, inosine, and

1-methyl adenine in this lab, but the Rf values or absorbance spectra did not coincide. Unknown #1 was also compared with all the minor base spectra presented in Venkstern and Baev (1975) and none of these appeared to be the correct known. However, an extensive comparison of all minor bases will have to be made in this laboratory.

The incorporation of the isotope is very high in all three unknowns, in fact, higher than any of the known bases, but the importance of this cannot be evaluated at this time.

An extensive study to define the many growth factors necessary to grow Entamoeba axenically was attempted by Nakamura and Baker (1956). One group of substances they added to the modified Boeck-Drbohlau egg slant medium overlaid with horse-serum-Ringer's solution was components of nucleic acid metabolism. With this medium it was found that the combination of all five nitrogenous bases stimulated growth the best followed by R-5-P + ATP > cytosine > thymine > R-5-P > guanine adenine > adenylic acid, and finally ATP.

Later, methylthioadenosine was found to have a greater stimulatory effect than ATP or R-5-P when added to the basal medium. The addition of adenosine to the methylthioadensine containing medium did not increase the growth of this ameba; therefore, its stimulatory

activity is not due to the release of adenosine (Nakamura, 1957). Nakamura and Jonsson (1957) proceeded to look at the effects of analogs of purines and pyrimidines on growth. Many analogs had a negative effect on growth. Analogs such as 1-dichloroacetamido-2-nitro-4-methoxybenzene, 8-azaguanine, and 3,7-dimethylxanthine resulted in an inhibition which could not be reversed. Reversal could be seen with 2-dichloracetamido-2,6-dimethylpyrimidine, 2-aminopyrimidine, and uracil 5-carboxylic acid, as a few examples.

Reeves and West (1979) studied the needs of nucleic acid precursors by Entamoeba grown axenically in Diamond's TP-S-1 medium which was made flavin deficient. Their results indicated that the addition of adenosine showed the best growth. Adenosine being followed by adenine > AMP > AMP + CMP > AMP + GMP + UMP + CMP.

The results of the present study, using unmodified TP-S-1 medium were slightly different from the two reported above. AMP was found to be the one mononucleotide which stimulated growth the best. In order of decreasing stimulatory effect adenine > GMP > CMP > UMP followed.

All three studies indicated that adenine or an adenine derivative produced a good growth response. This is not too surprising because of the high demand of any cell for ATP. The fact that there was some

discrepancy as to the order in which any of these molecules improves growth may just be the result of the different experimental procedures. From the data presented in this study, one possible reason for the need of these compounds in the media is a replacement for the gaseous CO_2.

Normally when E. histolytica is cultivated no concern for adjusting the gas concentration in the air phase above the media is made. Band and Cirrito (1979) discovered that amebae grown in their 15 ml rubber sealed capped test-tubes containing 10 ml of medium will produce CO_2 at a concentration of 3 percent within 6 hours, in the air phase. Since this CO_2 is not removed during standard cultivation, it could easily satisfy any gas requirement. Therefore, its importance in any interactions with nucleic acid, protein, or lipid biosynthesis could be overlooked.

Booden et al. (1976) looked at the incorporation of tritium-labeled purines and pyrimidines into E. histolytica over a 24 hour period. The results indicate that both purines and pyrimidines gained access inside the cell. Peak time of incorporation, and the concentration taken up by the cell varied with the nitrogeneous base. Adenine had an incorporation of 4.3 x 10^5 cpm/10^6 amebae, followed by adenosine (3.8 x 10^5 cmp/10^6 amebae); but there is no significant

preference of either one by the cell. Guanosine is the next preferred nucleic acid precursor to be taken up by the cell. Its incorporation is 2.5 fold greater than guanine.

Purines enter the cell at higher concentration than pyrimidines. Cytidine (2.4×10^5 cpm/10^6 amebae) is the most extensively incorporated pyrimidine derivative, followed by uridine > uracil > thymidine > cytosine > thymine.

The study of the uptake mechanism of purine bases and their nucleosides was studied by Boonlayangoor et al. (1978). Based on saturation kinetics, competitive homologs and analogs, diffusion and inhibitable components, and Q_{10} values > 2, they found that adenine, adenosine, and guanosine were taken up, in part, by a "carrier-mediated" system. Guanine, hypoxanthine and inosine enter the cell via diffusion. Individual transport sites for adenine-adenosine and adenosine-guanosine are supported by the inhibitor studies. The "non-productive" binding experiments involving guanine hypoxanthine, and inosine gave evidence of additional sites of transport of adenine, adenosine, and guanosine. Also uptake of adenine, adenosine, and guanosine was reduced by iodoacetate and N-ethylmaleimide.

In view of the fact that the rate of incorporation of the various nucleosides, nucleotides, and

nitrogenous bases is different, this would certainly affect their ability to spare CO_2. Also the rate of incorporation of adenosine, guanosine, cytidine, and uridine is in the same order as their phosphorylated forms stimulate growth in the absence of CO_2.

SUMMARY

Entamoeba histolytica's growth improved when grown axenically in Diamond's TP-S-1 medium with the addition of exogenous nitrogenous bases, nucleosides or nucleotides in the presence of 10 percent carbon dioxide in the air phase above the medium. It was shown that AMP stimulates growth the best, when added at a final concentration of 1.25 µmoles in the absence of CO_2. Adenine, GMP, CMP and UMP also spared CO_2 but to a lesser degree.

The rate of uptake of the various purines and pyrimidines varies. Part of this difference is due to the different types of transport systems used by *Entamoeba* to get these molecules into the cell. The ability of any of these molecules to spare CO_2 is identical to its preference of uptake by the cell. Adenine and adenosine are taken up in the highest concentrations by the ameba, and it is adenine and its derivatives which stimulate growth the best.

Because the CO_2 molecule is known to be incorporated into the ring structure of purines and pyrimidines of many cells, the incorporation of this molecule was studied here in *Entamoeba*. Both the purines and the

pyrimidines incorporated CO_2, except thymine. There may also be incorporation into thymine, but, because the concentration of thymine in the cell is so small, the incorporation of ^{14}C-sodium bicarbonate into thymine could not be quantified by the methods used. The highest uptake of the isotope was concentrated in adenine.

Three spots which were identified with two dimensional TLC were noticed. Only one spot, unknown #1, produced a peak around 260 nm. Its properties were compared with several minor bases, and various intermediates in the synthesis of purines and pyrimidines, but no identity was made. These three spots incorporated the isotope to a higher degree than any of the other nitrogenous bases.

^{14}C-sodium bicarbonate was also incorporated into the ethanol-ether soluble, and the hot TCA in soluble fractions. An attempt was made to identify the various amino acids which took up the isotope, but the level of activity was too low for detection under these authors' conditions. The ethanol-ether soluble fraction also showed some incorporation of sodium bicarbonate, but the importance of this incorporation into lipids was not pursued at this time.

Many other rumen microorganisms have a carbon dioxide requirement. It seems only logical that Entamoeba, also found in the gut, would also have the

same requirement. It is known that *E. coli* and *Streptococcus bovis*, as two examples, need CO_2 in the synthesis of proteins, nucleic acids and lipids, and this appears to be the case in the synthesis of nucleic acids by *Entamoeba*.

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