ABSORPTION OF THE NATURAL PURINES AND PYRIMIDINES BY THE GUT OF THE CHITON, CRYPTOCHITON STELLERI

A Thesis

Presented to

the Faculty of the Department of Biology

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

---

by

Martin Edward Hanisch

May 1970

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ABSTRACT

The everted sac technique was utilized to study the absorption of three pyrimidines (thymine, uracil, and cytosine) and four purines (guanine, hypoxanthine, adenine, and uric acid) in the gut of the primitive mollusc Cryptochiton stelleri.

Thymine was shown to be actively transported in a concentration range of 0.156-15.60 nM/ml. Saturation kinetics were not observed at the highest concentration of thymine used. Thymine and guanine were actively transported in all regions of the chiton gut tested. Uracil was actively transported only in the proximal anterior gut, whereas hypoxanthine was actively transported only in the posterior gut regions. Cytosine, adenine, and uric acid displayed only fractional S/M ratios and negative net serosal accumulation values which indicated that these purines and pyrimidines were not actively transported.

Studies using anaerobic conditions or DNP indicated that the active transport mechanism for the purines and pyrimidines in the chiton gut was dependent on a constant supply of metabolic energy.

The active transport of the nitrogenous bases by the gut of the chiton displayed the following similarities to the mammalian active transport mechanisms:

1. Thymine, uracil, guanine, and hypoxanthine are moved against their apparent concentration gradients.

2. Active transport is dependent on an aerobic metabolic energy supply.

3. Purines and pyrimidines are not metabolized during absorption.

4. Specificity is displayed in different regions of the gut of the chiton and for different purines and pyrimidines.

The following differences were observed in the active transport of the nitrogenous bases in the chiton from that displayed in the mammalian gut:

1. The purines hypoxanthine and guanine are actively transported in the chiton gut.

2. The active transport of hypoxanthine is less sensitive to DNP in the chiton.

3. The gut is capable of utilizing its active transport mechanism after prolonged anaerobic conditions.

4. Larger S/M ratios are developed with the active transport mechanism in the gut of the chiton.

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INTRODUCTION

Evidence for the absorption of organic substances by means other than passive diffusion was obtained from studies performed from 1900 to 1930. The mechanisms involved in the absorption process were difficult to characterize due to lack of adequate equipment and sophisticated procedures necessary for the characterization of the absorption process.

With the advent of modern techniques, the active transport system is now characterized by the following criteria:

1. The substance must have the capability to move against its apparent concentration and/or electrical gradient.
2. The mechanism must require an expenditure of metabolic energy.
3. The substance must not be metabolized in its passage through the intestinal wall.
4. The process must display some degree of specificity.

Most active transport studies of organic substances have been primarily concerned with absorption of amino acids, monosaccharides, and fatty acids. Several excellent reviews have been published concerning the vast information on the active transport of amino acids, monosaccharides, and fatty acids (Benson and Rampone, 1966; Wilson, 1962; Wiseman, 1964; Crane, 1960; Smyth and Whittam, 1967). The following summarizes the comparatively small amount of knowledge concerned with the active transport of purines and pyrimidines

(nitrogenous bases) by animal guts.

The first studies concerning the absorption of purines and pyrimidines were conducted by Mendel and Myers (1910). Ingested cytosine, thymine, and uracil were shown to be absorbed by the intestine of rabbits and men.

However, there were no further studies which provided data pertaining to the absorption of the nitrogenous bases until the 1940's. The following exogenous purines and pyrimidines were shown to be absorbed but not to be used as metabolic precursors for the synthesis of purines and pyrimidines present in the nucleic acids in rats: guanine (Plentl and Schoenheimer, 1944); uracil (Plentl and Schoenheimer, 1944); thymine (Plentl and Schoenheimer, 1944); cytosine (Bendich, et al., 1949); hypoxanthine (Getler, et al., 1949); and xanthine (Getler, et al., 1949). However, Brown, et al. (1948) demonstrated that ingested adenine was absorbed and incorporated into tissue nucleic acids, muscle ATP, and was a metabolic precursor to guanine in the rat.

In digestive studies conducted by Wilson and Wilson (1958) it was observed that pyrimidine nucleotides were hydrolyzed in the gut of the rat to their respective nucleosides and free bases. However, neither the nucleosides nor their nitrogenous bases were absorbed against their concentration gradients. In similar experiments, purine nucleotides were hydrolyzed to their respective nucleosides and

inorganic phosphate. The resulting nucleosides were than absorbed and incorporated into the body nucleic acids (Wilson and Wilson, 1962). In the same studies, when the purines, guanine, xanthine, and hypoxanthine were absorbed, they were catabolized to waste products and excreted.

The first evidence for the presence of an active transport mechanism for a nitrogenous base was substantiated by Schanker and Tocco in 1960. They obtained evidence for the active transport of uracil and thymine by demonstrating saturation kinetics and competitive inhibition by similar compounds (e.g. hypoxanthine, cytosine, 6-azauracil, 6-azathymine). Furthermore, Schanker and Jeffrey (1961) reported that the foreign pyrimidines, 5'-fluorouracil and 5'-bromouracil, were also actively transported and were competitive inhibitors of uracil active transport. In a later study, Schanker and Tocco (1962) showed that active transport of uracil also was found in the gut of the rat, hamster, and frog. The absorption process required oxygen, was blocked by various metabolic inhibitors, and was inhibited by pyrimidines which have structural characteristics similar to the uracil molecule. In 1962, Schanker and Jeffrey studied the structural requirements of the pyrimidines which inhibited the active transport of uracil. They observed that strong inhibition was displayed by the simple derivatives of uracil having a substituent replacing the hydrogen atom at the

carbon in the five or six position. Whereas changes in almost any other portion of the molecule yielded compounds which displayed weak or insignificant inhibitory activity on the active transport of uracil. Schanker, Jeffrey, and Tocco (1963) found that the purines hypoxanthine, xanthine, uric acid, 6-mercaptopurine, and guanine inhibited uracil transport by 30% to 100%. Adenine, 3-methylxanthine, theophylline, theobromine, and caffeine showed no inhibitory effect on the active transport of uracil.

Uric acid has been shown to be excreted only by passive diffusion in the small intestine of the rat (Oh, Dossetor, and Beck, 1967).

In a very recent study(Berlin and Hawkins, 1968a, b), hypoxanthine and xanthine appeared to be actively transported (secreted) into the lumen of the small intestine in rats. Adenine was not secreted but was passively absorbed and incorporated directly into body nucleic acids. Guanine was rapidly deaminated in the intestinal wall. The active transport of purines yielded net fluxes approximately twice as high as those developed for the active transport of uracil by the gut, indicating a separate "carrier" system for the purines. The active transport of the purines was inhibited by 2,4-dinitrophenol (DNP) and the secretory "pump" appeared to be located at the basal membrane of the epithelial cells of the small intestine. The active transport of

the purines was also independent of the Na^+ concentration and was ouabain insensitive. This was in contrast to the observation by Csáky (1961), that the active transport of uracil was dependent on the Na^+ concentration.

Very few studies have been conducted to test for the active transport of nitrogenous bases in invertebrate animals. Macinnis, Fisher, and Read (1965) obtained data showing that the rat cestode Hymenolepsis dimunita had at least one locus for the active transport of purines and pyrimidines. Hypoxanthine, uracil and adenine had affinities for the system and reciprocal competitive inhibition between purines and pyrimidines occurred. Thymine and cytosine displayed no affinity for the active transport system but were absorbed by simple diffusion. The active transport system had a different locus than that for monosaccharide or amino acid active transport. An active transport mechanism was also shown to be present in Escherichia coli for the active transport of adenosine (Peterson and Koch, 1966). The mechanism was saturable, could be inhibited by other nucleosides and metabolic poisons, and was not inhibited by free bases.

Since there have been no studies to determine if active transport of purines or pyrimidines occurred in the gut of any marine invertebrate, it was of great interest to:

1. Determine if the free bases are moved against their concentration gradients.

2. Determine if the absorption process requires aerobic conditions.

3. Determine if the free bases are metabolized as they are absorbed.

4. Determine if the "carrier" displays some degree of specificity as to which compounds are actively transported and in which regions of the gut.

5. Compare the active transport mechanism of this marine invertebrate with that of the vertebrate gut.

MATERIALS AND METHODS

Animals

Adult starved chitons (*Cryptochiton stelleri*) of both sexes were used in this investigation. Chitons were provided by Pacific Bio-marine Supply Company, Venice, California. The chitons were kept in aerated and filtered sea water at 5°C for several days prior to their use in the experiments.

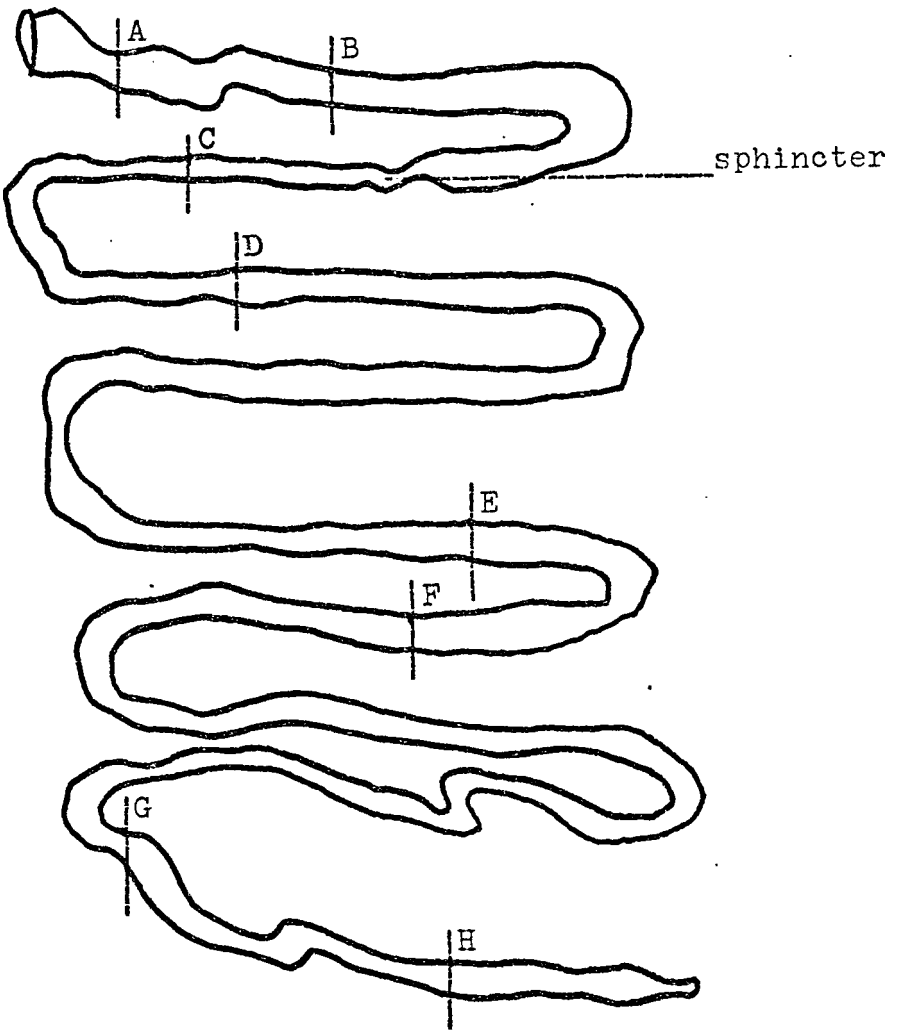
Isolation of gut segments

The animals were sacrificed, the visceral masses removed, and the guts separated from the visceral masses. The proximal portion of the anterior gut and segments from the proximal, medial, and distal regions of the posterior gut (Figure 1) were used in this investigation. The proximal posterior segments were taken from the region of the gut starting 4-6 cm distal to the sphincter separating the anterior and the posterior intestine; the medial posterior segments were secured from the middle region of the posterior intestine; and the distal posterior segments were obtained from the region proximal to the terminal 6 cm portion of the posterior intestine. The anterior gut segments were 2-3 cm in length, and the posterior gut segments were 6-10 cm in length.

In vitro method

The experimental design used was the *in vitro* everted

FIGURE 1 ISOLATED C. stelleri GUT



Regions of gut are as follows:

A-B: proximal anterior

C-D: proximal posterior

E-F: medial posterior

G-H: distal posterior

sac method of Crane and Wilson (1958) as modified by Lawrence (1962). The following procedure was used in everting the gut segments. A small glass rod was inserted through the lumen of the isolated gut segment. After one end of the gut segment was ligated to the end of the rod, the free end of the gut segment was pulled back over the rod exposing the mucosal surface of the segment. One end of the everted gut segment was then tied to a small glass weight, and the opposite end of the segment was ligated to the tip of a small bore glass cannula. Figure 2 shows the experimental design which was employed.

The everted gut segment was then immersed in a wide-mouth test tube which contained 32 ml of chiton Ringer solution (Table 1) containing a known amount of ^{14}C-labeled purine or pyrimidine. From 0.2-1.7 ml of the same solution was pipetted into the serosal sac through the cannula. The fluid contained by the sac was termed the serosal fluid, and that fluid contained by the test tube was designated the mucosal fluid. All experiments were performed at a temperature of 15°C by using a Magni-Whirl Model MR-3240D constant temperature water bath. The mucosal fluid was continuously aerated by bubbling 10-20 ml of atmospheric air/minute through it.

At the end of a preincubation or test period, the serosal fluid was removed from the everted sac with a small pi-

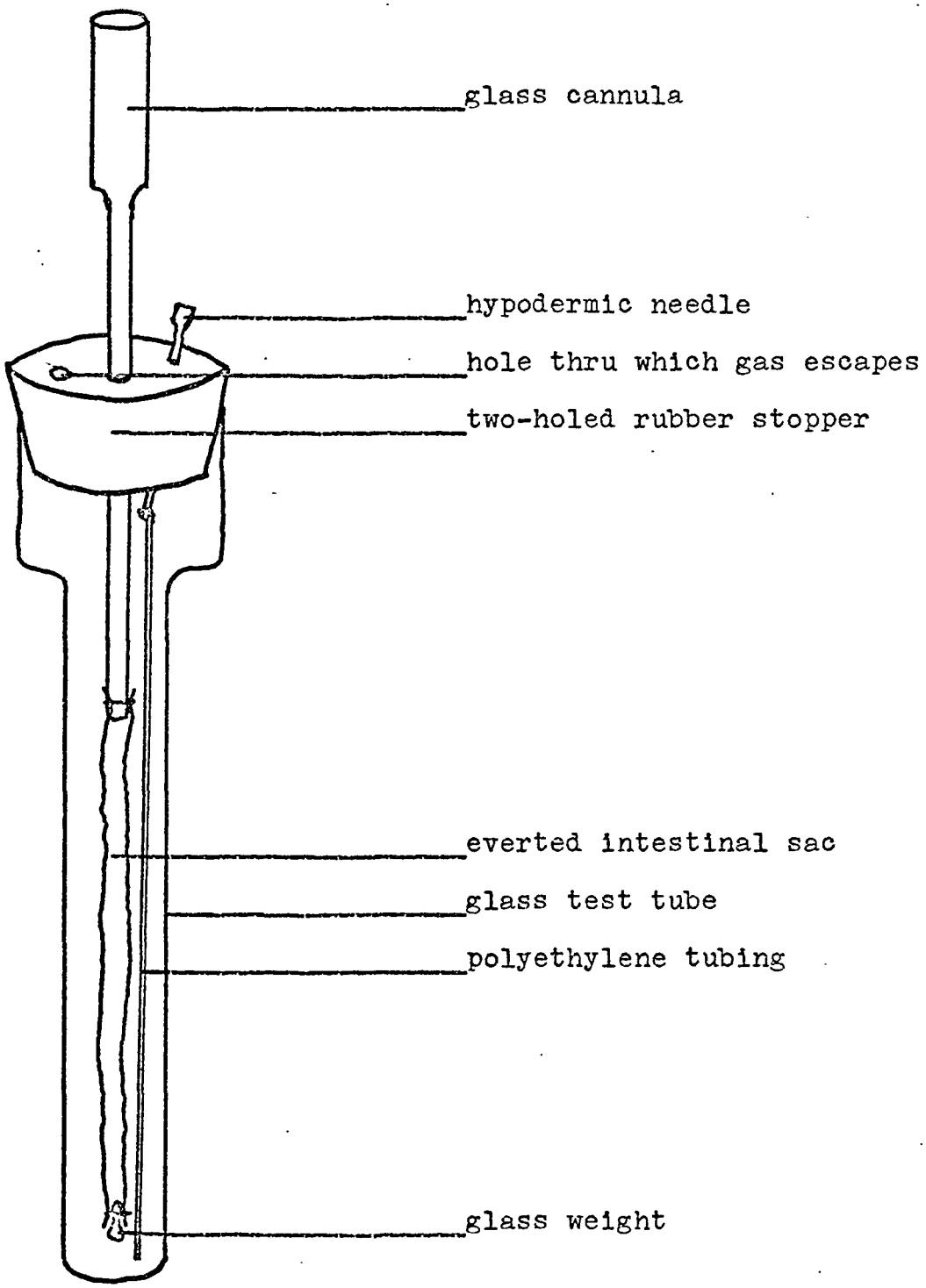


FIGURE 2: Apparatus Used in the <u>in vitro</u> Method for the Study of Absorption of Purines and Pyrimidines

TABLE 1 COMPOSITION OF "CHITON RINGER" SOLUTION USED IN TRANSPORT STUDIES

Compound	Grams/liter	mMoles
NaCl	27.0	462.00
KCl	0.9	12.07
$MgCl_2$	2.0	9.83
$MgSO_4$	0.6	2.43
$NaHCO_3$	0.2	2.38
$CaCl_2$	1.5	13.32
Glucose	0.033	0.202

pet through the glass cannula. The cannula and the attached empty segment were then immediately placed into another wide-mouth test tube containing the desired solution for the next preincubation or test period. A desired new solution was then pipetted into the serosal compartment. When the last test period was completed, the serosal fluid was removed and the gut segment detached from the cannula and glass weight and immediately placed into 8 ml of 80% ethanol solution. A sample of the mucosal solution also was taken at the end of each test period.

Chemicals

The following radioisotopes used in these studies were obtained from New England Nuclear Corporation: thymine-2-^{14}C, uracil-2-^{14}C, cytosine-2-^{14}C, adenine-8-^{14}C, and hypoxanthine-8-^{14}C. Uric acid-2-^{14}C and guanine-8-^{14}C were obtained from Volk Radiochemical Company. All of the other chemicals used in these studies were of reagent grade and were obtained from commercial sources.

Chromatography

One dimensional descending paper chromatography was used in determining if the nitrogenous bases were chemically altered as they were absorbed. The solvent which was used was 5% K_2HPO_4 in distilled H_2O. Two 0.01 ml aliquots of the final serosal fluid samples were spotted on Whatman #1 filter paper. On these spots were spotted a 0.01 ml aliquot of

the test purine or pyrimidine (1.0 mg/ml) which was used as a tracer so the developed spot would fluoresce under ultraviolet light. The fluorescence indicated where the test purine or pyrimidine was located.

The developed spots were placed in 15 ml of cocktail I (6 gm PPO, 100 gm naphthalene, 100 ml H_2O, make up to 1000 ml with 1,4-dioxane) and counted in a liquid scintillation counter. The radioactivity of these spots were compared to control values obtained from similar spots which had not been developed. The difference in radioactivity between the control values and the developed spots was interpreted to be that fraction of the nitrogenous base which had been metabolized as it was transported across the gut.

Radioactive determinations

Samples (0.1 ml) of final serosal and mucosal fluids were placed in 15 ml of cocktail I and counted in a Packard Tricarb Model 3003 Liquid Scintillation Spectrometer or a Beckman Liquid Scintillation Counter to determine the amount of ^{14}C-labeled purine or pyrimidine present.

To determine the amount of test base accumulated into the gut tissue, a two-week old, 1 ml aliquot of ethanol extraction solution was added to 15 ml of cocktail I and counted in a liquid scintillation counter. The gut segments were divided into two portions which were individually weighed. One of these portions was dryed in an oven at 100°C for 24

hours and weighed. The other portion was solubilized in 1 ml of Soluene (Packard) to which was added 10 ml of cocktail II (6gm PPO/1000 ml toluene). This was counted in a liquid scintillation counter to determine the amount of the base incorporated into the ethanol insoluble portion of the intestinal tissue. It was assumed that the ^{14}C-label found in the ethanol soluble and insoluble fractions was the test purine or pyrimidine.

Calculations

The final serosal/mucosal ratio (S/M), the final net serosal accumulation, and tissue accumulation of the test nitrogenous bases were the parameters measured to determine if they were actively transported. The S/M ratio was calculated by dividing the final serosal by the final mucosal concentration. The final net serosal accumulation (nM/100 mg dry gut weight) was determined by subtracting the initial serosal concentration from the final serosal concentration and dividing by the dry weight of the gut segments. The total tissue accumulation and the ethanol soluble tissue accumulation were calculated. The ethanol soluble nitrogenous base/ml tissue H_2O plus the ethanol insoluble nitrogenous base/ml tissue H_2O was the total tissue accumulation expressed in nM/ml tissue H_2O.

Series I Concentration study with thymine

The *in vitro* method previously described was employed

in this study. Segments from the proximal anterior intestine and from the medial posterior intestine were used in this study. After an initial two-hour preincubation period, which was used to allow the gut to equilibrate, three consecutive two-hour test periods were done. An identical solution was used in the preincubation period as was used in the initial test period which followed.

Two experimental approaches were used in this study. In the first approach the initial thymine concentration used for the preincubation period and the first test period was 15.60 nM/ml, the second test period was 1.56 nM/ml, and the third test period was 0.156 nM/ml. In the second approach the concentrations were changed in the reverse order.

Series II Mapping determinations

The procedure used for mapping determinations of various nitrogenous bases was identical to that used in the thymine concentration study with the following exceptions.

1. After a two-hour preincubation period, a single six-hour test period was used except for uric acid with which a single four-hour test period was utilized.
2. The following initial serosal and mucosal concentrations were used: thymine 1.56 nM/ml; guanine 0.65 nM/ml; cytosine 1.56 nM/ml; uracil 1.56 nM/ml; adenine 1.56 and 0.65 nM/ml; hypoxanthine 0.65 nM/ml; and uric acid 0.38 nM/ml.
3. Allopurinol (13.0 nM/ml) was used in the experiments performed with hypoxanthine. The purpose was to block the xan-

thine oxidase activity.

4. Non-everted segments of intestine were used along with everted segments to determine if the purines were secreted.

Series III Inhibition studies

The same procedure was employed in these studies as was used in the thymine concentration study with the following differences. The only segments used were those from regions of the gut which showed maximal active transport of a nitrogenous base in the mapping determinations. Segments from the proximal anterior intestine were used with thymine, uracil, and guanine. Hypoxanthine, guanine, and thymine were tested using segments from the medial posterior intestine. A series of three two-hour test periods was used in these studies. The test periods were preceded by a preincubation period of two hours, one-half hour, and one-half hour respectively. The first and third test period and their respective preincubation periods were performed aerobically by bubbling air through the mucosal solution. The second test period and its preincubation period were carried out under anaerobic conditions maintained by bubbling purified nitrogen gas through the mucosal solution or by using dinitrophenol (1.0 nM/ml). At the end of each test period samples were collected from the serosal and mucosal fluid compartments as previously described.

RESULTS

The purines and pyrimidines which were actively transported as well as those not actively transported were shown not to be metabolized by the gut. This was demonstrated in that the final serosal concentration of the test purine or pyrimidine was 100±5% of the concentration in undeveloped spots by use of one-dimensional paper chromatography.

The dry weights of the chiton gut segments used in these studies varied from 12-67 mg. By measuring the terminal serosal sac volume at the end of the test periods, it was shown that no net water movement occurred.

When the ethanol soluble tissue accumulation values were significantly higher ($P<.05$) than the initial ambient concentration of the test purine or pyrimidine, this was used as an index of active transport.

Series I Concentration study with thymine

Table 2 shows the effect of concentration on thymine active transport in the proximal anterior intestine and the medial posterior intestine. As indicated in this table, positive S/M ratios (>1.00) were developed at all three concentrations used. A positive net serosal accumulation also was observed in all of the experiments. The highest relative net serosal accumulation in the proximal anterior intestine was obtained using 0.156 nM of thymine per ml. Total net tissue accumulation and ethanol soluble tissue accu-

TABLE 2 THE EFFECT OF CONCENTRATION ON THYMINE ACTIVE TRANSPORT IN C.stelleri GUT

Init. conc. (nM/ml)	S/M ratio	Final serosal concentration (nM/ml)	Net serosal accumulation (nM/100 mg dry gut weight)	Total net tissue accumulation (nM/ml tissue water)	Ethanol soluble accumulation(nM/ml tissue water)
			Proximal Anterior Intestine		
15.60	8.09 ± 0.99	39.28 ± 1.82	54.64 ± 2.05	38.85 ± 5.37	29.35 ± 3.90
1.56	7.09 ± 0.88	10.06 ± 1.88	16.26 ± 2.53	13.09 ± 1.51	9.07 ± 0.67
0.156	7.02 ± 0.54	0.921 ± 0.075	1.56 ± 0.19	1.80 ± 0.33	1.29 ± 0.23
			Medial Posterior Intestine		
15.60	18.93 ± 1.70	65.05 ± 4.74	121.94 ± 9.48	38.08 ± 4.78	30.03 ± 3.65
1.56	10.85 ± 0.92	14.20 ± 1.20	30.54 ± 2.59	16.45 ± 2.34	12.55 ± 1.77
0.156	8.38 ± 1.30	1.131 ± 0.103	2.90 ± 0.22	2.90 ± 1.35	2.16 ± 1.01

Everted segments were incubated aerobically for 3 consecutive 2 hour test periods. The initial serosal and mucosal concentrations of thymine were equal. Each value represents the mean of four observations ± S.E.M.

mulation/ml tissue water of thymine were significantly higher ($P<.05$) than the initial concentration of thymine in the incubation media.

Series II Mapping determinations

The absorption of the pyrimidines thymine, uracil, and cytosine by different regions of the gut can be seen in Tables 3, 4, and 5. Thymine was actively transported as evidenced by positive S/M ratios, positive net serosal accumulation, and ethanol soluble tissue accumulation values in all regions tested in the gut (Table 3). The greatest accumulation of thymine into the serosal compartment and gut tissue occurred in the proximal anterior and medial posterior regions of the gut. However, there was a significant increase ($P<.05$) in tissue accumulation over ambient concentration of uracil and positive S/M ratios only in the proximal anterior gut (Table 4). Fractional S/M ratios and negative net serosal accumulation values were observed in all gut regions when cytosine was the test pyrimidine (Table 5). The total net tissue accumulation did not differ significantly ($P>.05$) from the initial cytosine concentration in the incubation media.

Of the purines tested (Tables 6, 7, 8, and 9), only guanine and hypoxanthine were actively transported as indicated by positive S/M ratios, net serosal accumulation, and tissue accumulation significantly higher than initial am-

TABLE 3 ABSORPTION OF THYMINE BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	2.62 ± 0.48	1.45 ± 0.48	3.10 ± 0.73	1.47 ± 0.06
Final serosal concentration (nM/ml)	3.58 ± 0.57	2.13 ± 0.28	4.29 ± 0.77	2.08 ± 0.07
Net serosal accumulation (nM/100 mg dry gut weight)	4.71 ± 0.75	0.65 ± 0.08	4.48 ± 0.80	1.03 ± 0.04
Total net tissue accumulation (nM/ml tissue water)	11.08 ± 1.28	8.94 ± 0.84	16.51 ± 2.22	10.68 ± 1.83
Ethanol soluble accumulation (nM/ml tissue water)	8.05 ± 0.93	6.86 ± 0.64	12.13 ± 1.63	8.14 ± 1.44

Everted segments were incubated aerobically for 6 hours. The initial serosal and mucosal thymine concentrations were 1.56 nM/ml. Each value represents the mean of four observations ± S.E.M.

TABLE 4 ABSORPTION OF URACIL BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	2.58 ± 0.40	0.83 ± 0.04	0.85 ± 0.25	0.91 ± 0.42
Final serosal concentration (nM/ml)	3.49 ± 0.41	1.25 ± 0.04	0.87 ± 0.13	1.11 ± 0.44
Net serosal accumulation (nM/100 mg dry gut weight)	4.84 ± 0.56	-0.43 ± 0.01	-1.42 ± 0.21	-1.01 ± 0.32
Total net tissue accumulation (nM/ml tissue water)	13.71 ± 1.82	1.02 ± 0.28	1.62 ± 0.13	1.64 ± 0.20
Ethanol soluble accumulation (nM/ml tissue water)	10.64 ± 1.39	0.77 ± 0.21	1.24 ± 0.18	1.19 ± 0.16

Everted segments were incubated aerobically for 6 hours. The initial serosal and mucosal uracil concentrations were 1.56 nM/ml. Each value represents the mean of six observations ± S.E.M.
- indicates net loss.

TABLE 5 ABSORPTION OF CYTOSINE BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	0.87 ± 0.03	0.88 ± 0.02	0.85 ± 0.25	0.91 ± 0.42
Final serosal concentration (nM/ml)	1.53 ± 0.26	1.41 ± 0.04	1.37 ± 0.05	1.46 ± 0.01
Net serosal accumulation (nM/100 mg dry gut weight)	-0.05 ± 0.01	-0.17 ± 0.01	-0.20 ± 0.07	-0.16 ± 0.00
Total net tissue accumulation (nM/ml tissue water)	2.09 ± 1.01	1.57 ± 0.08	1.80 ± 0.45	1.66 ± 0.83
Ethanol soluble accumulation (nM/ml tissue water)	1.52 ± 0.78	1.13 ± 0.06	1.36 ± 0.30	1.20 ± 0.60

Everted segments were incubated aerobically for 6 hours. The initial serosal and mucosal cytosine concentrations were 1.56 nM/ml. Each value represents the mean of four observations ± S.E.M.
- indicates net loss.

TABLE 6 ABSORPTION OF GUANINE BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	1.58 ± 0.04	10.90 ± 4.40	65.10 ± 1.98	3.40 ± 0.69
Final serosal concentration (nM/ml)	0.92 ± 0.01	5.21 ± 1.70	6.19 ± 1.02	1.45 ± 0.22
Net serosal accumulation (nM/100 mg dry gut weight)	0.61 ± 0.01	8.37 ± 2.73	8.70 ± 1.43	0.47 ± 0.07
Total net tissue accumulation (nM/ml tissue water)	0.82 ± 0.14	3.18 ± 0.40	3.04 ± 0.42	2.27 ± 0.32
Ethanol soluble accumulation (nM/ml tissue water)	0.60 ± 0.05	2.32 ± 0.31	2.21 ± 0.31	1.68 ± 0.24

Everted segments were incubated aerobically for 6 hours. The initial serosal and mucosal guanine concentrations were 0.65 nM/ml. Each value represents the mean of two observations ± S.E.M.

TABLE 7 ABSORPTION OF HYPOXANTHINE BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	0.53 ± 0.11	14.90 ± 3.53	85.20 ± 21.08	10.32 ± 1.42
Final serosal concentration (nM/ml)	0.27 ± 0.01	2.58 ± 1.17	6.81 ± 11.55	2.73 ± 0.36
Net serosal accumulation (nM/100 mg dry gut weight)	-0.49 ± 0.02	1.39 ± 0.63	6.31 ± 1.41	2.76 ± 0.37
Total net tissue accumulation (nM/ml tissue water)	0.63 ± 0.15	12.29 ± 2.84	14.51 ± 2.72	12.26 ± 1.24
Ethanol soluble accumulation (nM/ml tissue water)	0.47 ± 0.13	9.96 ± 2.31	10.54 ± 1.67	10.12 ± 1.02

Everted segments were incubated aerobically for 6 hours. The initial serosal and mucosal hypoxanthine concentrations were 0.65 nM/ml. Each value represents the mean of two observations ± S.E.M. - indicates net loss.

TABLE 8 ABSORPTION OF ADENINE BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	0.28 ± 0.05	0.41 ± 0.04	0.25 ± 0.03	0.23 ± 0.02
Final serosal concentration (nM/ml)	0.19 ± 0.04	0.28 ± 0.01	0.17 ± 0.03	0.14 ± 0.02
Net serosal accumulation (nM/100 mg dry gut weight)	-1.05 ± 0.22	-0.64 ± 0.03	-1.00 ± 0.19	-1.16 ± 0.16
Total net tissue accumulation (nM/ml tissue water)	0.61 ± 0.08	0.65 ± 0.21	0.46 ± 0.18	0.62 ± 0.07
Ethanol soluble accumulation (nM/ml tissue water)	0.47 ± 0.07	0.51 ± 0.15	0.36 ± 0.06	0.50 ± 0.08

Everted segments were incubated aerobically for 6 hours. The initial serosal and mucosal adenine concentrations were 0.65 nM/ml. Each value represents the mean of two observations ± S.E.M.
- indicates net loss.

TABLE 9 ABSORPTION OF URIC ACID BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	0.92 ± 0.02	0.95 ± 0.02	0.98 ± 0.03	0.95 ± 0.02
Final serosal concentration (nM/ml)	0.35 ± 0.02	0.35 ± 0.00	0.37 ± 0.01	0.35 ± 0.01
Net serosal accumulation (nM/100 mg dry gut weight)	-0.05 ± 0.00	-0.04 ± 0.00	-0.01 ± 0.00	-0.04 ± 0.00
Total net tissue accumulation (nM/ml tissue water)	0.37 ± 0.05	0.35 ± 0.03	0.33 ± 0.02	0.29 ± 0.03
Ethanol soluble accumulation (nM/ml tissue water)	0.29 ± 0.03	0.26 ± 0.02	0.25 ± 0.02	0.23 ± 0.03

Everted segments were incubated aerobically for 4 hours. The initial serosal and mucosal uric acid concentrations were 0.38 nM/ml. Each value represents the mean of four observations ± S.E.M.
- indicates net loss.

bient purine concentration ($P<.05$). Guanine was actively transported in all gut regions with maximal transport occurring in the medial posterior gut region (Table 6). Active transport of hypoxanthine as seen in Table 7 was observed in the three regions of the posterior gut, with maximal transport occurring again in the medial posterior gut region. Only fractional S/M ratios, negative net serosal accumulation, and tissue accumulation significantly lower than initial ambient concentration ($P<.05$) were obtained using adenine and uric acid as test compounds (Tables 8 and 9).

In those studies conducted with uneverted gut segments using guanine, hypoxanthine, and adenine (Tables 10, 11, and 12) positive S/M ratios were observed after 6 hours but a negative net serosal accumulation was seen. A greater net loss of activity in the mucosal fluid due to tissue accumulation and/or metabolism than in the serosal fluid accounted for the positive S/M ratios observed. Total net tissue accumulation/ml tissue water was significantly higher ($P<.05$) than initial incubation concentration of purines only in the proximal anterior gut region with guanine and in the regions of the posterior gut with guanine and hypoxanthine.

Series III Inhibition studies

As stated earlier, one of the requisites for an active transport mechanism is a constant supply of metabolic energy. By placing the preparation under anaerobic conditions, a sig-

TABLE 10 ABSORPTION OF GUANINE BY UNEVERTED GUT SEGMENTS BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	2.46 ± 0.09	1.52 ± 0.70	1.16 ± 1.06	2.12 ± 0.89
Final serosal concentration (nM/ml)	0.58 ± 0.03	0.55 ± 0.00	0.54 ± 0.01	0.52 ± 0.01
Net serosal accumulation (nM/100 mg dry gut weight)	-0.16 ± 0.01	-0.08 ± 0.00	-0.12 ± 0.00	-0.15 ± 0.00
Total net tissue accumulation (nM/ml tissue water)	1.51 ± 0.10	3.57 ± 0.91	3.86 ± 0.39	2.72 ± 0.31
Ethanol soluble accumulation (nM/ml tissue water)	1.18 ± 0.09	2.72 ± 0.73	3.08 ± 0.28	2.17 ± 0.26

Uneverted segments were incubated aerobically for 6 hours using initial equal serosal and mucosal concentrations (0.65 nM/ml) of guanine. Each value represents the mean of two observations ± S.E.M. - indicates net loss.

TABLE 11 ABSORPTION OF HYPOXANTHINE BY UNEVERTED GUT SEGMENTS BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	7.93 ± 0.04	2.20 ± 0.81	1.00 ± 0.01	1.05 ± 0.42
Final serosal concentration (nM/ml)	0.49 ± 0.02	0.52 ± 0.01	0.47 ± 0.02	0.43 ± 0.01
Net serosal accumulation (nM/100 mg dry gut weight)	-0.31 ± 0.01	-0.17 ± 0.00	-0.16 ± 0.01	-0.29 ± 0.00
Total net tissue accumulation (nM/ml tissue water)	0.71 ± 0.07	10.26 ± 2.85	12.67 ± 2.40	13.98 ± 1.82
Ethanol soluble accumulation (nM/ml tissue water)	0.54 ± 0.06	7.64 ± 2.39	9.15 ± 1.78	10.33 ± 1.61

Uneverted segments were incubated aerobically for 6 hours using initial equal serosal and mucosal concentrations (0.65 nM/ml) of hypoxanthine. Each value represents the mean of two observations ± S.E.M. - indicates net loss.

TABLE 12 ABSORPTION OF ADENINE BY UNEVERTED GUT SEGMENTS BY DIFFERENT REGIONS OF C. stelleri GUT

	Proximal anterior intestine	Proximal posterior intestine	Medial posterior intestine	Distal posterior intestine
S/M ratio	3.13 ± 0.06	2.44 ± 0.63	2.60 ± 0.17	2.78 ± 0.38
Final serosal concentration (nM/ml)	1.25 ± 0.22	1.07 ± 0.26	1.20 ± 0.08	0.90 ± 0.06
Net serosal accumulation (nM/100 mg dry gut weight)	-0.77 ± 0.13	-0.38 ± 0.09	-0.45 ± 0.03	-0.65 ± 0.04
Total net tissue accumulation (nM/ml tissue water)	1.44 ± 0.17	1.60 ± 0.17	1.51 ± 0.12	1.57 ± 0.32
Ethanol soluble accumulation (nM/ml tissue water)	1.06 ± 0.13	1.43 ± 0.30	1.30 ± 0.10	1.15 ± 0.24

Uneverted segments were incubated aerobically for 6 hours using initial equal serosal and mucosal concentrations (1.56 nM/ml) of adenine. Each value represents the mean of four observations ± S.E.M. - indicates net loss.

nificant reduction ($P<.05$) of the S/M ratios, final serosal concentration, and net serosal accumulation was noted in all cases of purines and pyrimidines actively transported (Tables 13, 14, 15, and 16). Significant increases ($P<.05$) of S/M ratios, final serosal concentration, and net serosal accumulation were observed with thymine, uracil, guanine in the medial posterior region, and hypoxanthine during reestablishment of the aerobic state. However, guanine (Table 15) in segments from the proximal anterior gut, upon returning to an aerobic state, did not increase the S/M ratios, final serosal concentration, or net serosal accumulation but decreased significantly ($P<.05$) from the levels observed in the anaerobic state. Observed net serosal accumulation and S/M ratios increased to at least their previous levels upon returning to the aerobic state with the exception of guanine (Table 15).

By adding DNP (1.0 uM/ml media), the S/M ratio, final serosal concentration, and final net serosal accumulation decreased, but not significantly ($P>.05$), the active transport of hypoxanthine in the medial posterior gut region (Table 17). Upon returning the preparation to an aerobic state, the transport was again increased, but not significantly ($P>.05$).

TABLE 13 EFFECT OF ANAEROBIC CONDITIONS OF ACTIVE TRANSPORT OF THYMINE IN C. stelleri GUT

Condition and number of observations	S/M ratio	Final serosal concentration (nM/ml)	Net serosal accumulation (nM/100 mg dry gut weight)	Total net tissue accumulation(nM/ ml tissue water)	Ethanol soluble accumulation(nM/ ml tissue water)
			Proximal Anterior Intestine		
aerobic (4)	1.73 ± 0.19	2.63 ± 0.27	1.87 ± 0.19	--	--
anaerobic (4)	1.51* ± 0.23	2.20* ± 0.25	1.13* ± 0.13	--	--
aerobic (4)	1.95* ± 0.21	2.96* ± 0.29	2.45* ± 0.24	13.09 ± 1.57	9.07 ± 0.65
			Medial Posterior Intestine		
aerobic (6)	8.41 ± 1.42	11.32 ± 1.72	13.21 ± 2.04	--	--
anaerobic (6)	4.17* ± 0.26	6.42* ± 1.05	6.60* ± 1.08	--	--
aerobic (6)	8.93* ± 0.62	13.85* ± 1.60	16.63* ± 1.92	16.45 ± 2.34	12.55 ± 1.77

The initial serosal and mucosal concentrations for all test periods were 1.56 nM thymine/ml. The duration of each test period was two hours. The experimental conditions of all test periods were identical with the exception that the second was under anaerobic conditions and the first and third under aerobic conditions. Each value represents a mean ± S.E.M.
* P ($<.05$) when compared to test period immediately preceding.

TABLE 14 EFFECT OF ANAEROBIC CONDITIONS OF ACTIVE TRANSPORT OF URACIL IN C. stelleri GUT

Condition and number of observations	S/M ratio	Final serosal concentration (nM/ml)	Net serosal accumulation (nM/100 mg dry gut weight)	Total net tissue accumulation (nM/ml tissue water)	Ethanol soluble accumulation(nM/ml tissue water)
			Proximal Anterior Intestine		
aerobic (4)	1.10 ± 0.06	1.75 ± 0.14	4.39 ± 0.60	--	--
anaerobic (4)	0.99* ± 0.04	1.52* ± 0.13	-0.09 ± 0.00	--	--
aerobic (4)	1.21* ± 0.12	1.85* ± 0.16	6.76* ± 0.59	11.02 ± 1.26	7.92 ± 0.93

The initial serosal and mucosal concentrations for all test periods were (1.56 nM/ml) of uracil. The duration of each test period was two hours. The experimental conditions of all test periods were identical with the exception that the second was under anaerobic conditions and the first and third under aerobic conditions. Each value represents a mean ± S.E.M.
* P ($<.05$) when compared to test period immediately preceding.
- indicates net loss.

TABLE 15 EFFECT OF ANAEROBIC CONDITIONS OF ACTIVE TRANSPORT OF GUANINE IN C. stelleri GUT

Condition and number of observations	S/M ratio	Final serosal concentration (nM/ml)	Net serosal accumulation (nM/100 mg dry gut weight)	Total net tissue accumulation(nM/ml tissue water)	Ethanol soluble accumulation(nM/ml tissue water)
			Proximal Anterior Intestine		
aerobic (7)	1.69 ± 0.14	1.05 ± 0.09	0.67 ± 0.06	--	--
anaerobic (7)	1.52* ± 0.08	0.86* ± 0.04	0.48* ± 0.02	--	--
aerobic (7)	0.90* ± 0.07	0.54* ± 0.04	-0.17* ± 0.01	0.86 ± 0.15	0.62 ± 0.10
			Medial Posterior Intestine		
aerobic (3)	5.24 ± 0.54	3.00 ± 0.29	2.14 ± 0.20	--	--
anaerobic (3)	3.21* ± 0.67	1.95* ± 0.36	1.18* ± 0.22	--	--
aerobic (3)	4.85* ± 0.14	2.56* ± 0.08	1.74* ± 0.05	2.09 ± 0.20	1.51 ± 0.17

The initial serosal and mucosal concentrations for all test periods were (0.65 nM/ml) of guanine. The duration of each test period was two hours. The experimental conditions of all test periods were identical with the exception that the second was under anaerobic conditions and the first and third under aerobic conditions. Each value represents a mean ± S.E.M.
* P ($<.05$) when compared to test period immediately preceding.
- indicates net loss.

TABLE 16 EFFECT OF ANAEROBIC CONDITIONS OF ACTIVE TRANSPORT OF HYPOXANTHINE IN C. stelleri GUT

Condition and number of observations	S/M ratio	Final serosal concentration (nM/ml)	Net serosal accumulation (nM/100 mg dry gut weight)	Total net tissue accumulation(nM/ml tissue water)	Ethanol soluble accumulation(nM/ml tissue water)
			Medial Posterior Intestine		
aerobic (3)	13.28 ± 3.03	6.56 ± 1.11	11.30 ± 1.90	--	--
anaerobic (3)	7.03* ± 1.60	4.09* ± 0.85	6.55* ± 1.36	--	--
aerobic (3)	18.79* ± 6.04	11.57* ± 5.71	20.80* ± 9.30	14.32 ± 2.69	10.85 ± 1.93

The initial serosal and mucosal concentrations for all test periods were (0.65 nM/ml) of hypoxanthine. The duration of each test period was two hours. The experimental conditions of all test periods were identical with the exception that the second was under anaerobic conditions and the first and third under aerobic conditions. Each value represents a mean ± S.E.M.

* P ($<.05$) when compared to test period immediately proceding.

TABLE 17 EFFECT OF DINITROPHENOL ON ACTIVE TRANSPORT OF HYPOXANTHINE IN C. stelleri GUT

Condition number of observations	S/M ratio	Final serosal concentration (nM/ml)	Net serosal accumulation (nM/100 mg dry gut weight)	Total net tissue accumulation(nM/ml tissue water)	Ethanol soluble accumulation(nM/ml tissue water)
			Medial Posterior Intestine		
Control (3)	6.99 ± 2.37	3.71 ± 0.93	3.57 ± 0.89	--	--
DNP (3)	5.73 ± 2.19	3.33 ± 1.05	3.12 ± 0.99	--	--
Control (3)	6.98 ± 2.87	3.61 ± 0.88	3.46 ± 0.85	14.70 ± 2.65	10.71 ± 2.01

The initial serosal and mucosal concentrations for all test periods were 0.65 nM hypoxanthine/ml. The duration of each test period was two hours. The experimental conditions of all test periods were identical with the exception that the second solution contained 1.0 nM DNP/ml and the first and third contained none. Each value represents a mean ± S.E.M.

DISCUSSION

The positive net serosal accumulation, S/M ratios>1.00, and net tissue accumulation values obtained with thymine, uracil, guanine, and hypoxanthine are evidence for the presence of an active transport system for the purines and pyrimidines. The fact that this active transport of these nitrogenous bases was dependent on a constant supply of aerobic metabolic energy demonstrated a similarity between the mammalian active transport for pyrimidines (Schanker and Tocco, 1962) and the active transport mechanism(s) displayed by the chiton for amino acids (Greer and Lawrence, 1967) and glucose (Lawrence and Lawrence, 1967). Further evidence for aerobic metabolic energy dependence was obtained by the return of S/M ratios and net serosal accumulation to the original control values or higher. This indicated that the inhibition observed under anaerobic conditions was not a result of tissue degeneration but due to the decreased aerobic metabolic energy supply.

The presence of more than one "carrier" system was suggested by these studies because of the observation that different nitrogenous bases are actively transported in different regions of the gut of the chiton.

At least two different "carriers" probably exist for the active transport of the pyrimidines. This statement is based on the fact that thymine was shown to be actively

transported in all regions of the gut tested, whereas uracil was actively transported only in the proximal anterior gut region. Two types of "carrier" systems are postulated:

1. A "carrier" exists in the posterior intestine which has an affinity to only thymine and a separate "carrier" in the anterior intestine which has an affinity for both uracil and thymine.
2. A separate "carrier" exists for thymine which is located in both the anterior and posterior intestine, whereas a separate "carrier" with an affinity for uracil is found only in the anterior intestine.

Unfortunately, from the data presented, either possibility is equally feasible. Cytosine was not actively transported and did not possess an affinity for the "carrier(s)" of thymine and uracil. This agrees with the transport of pyrimidines in mammalian systems (Schanker and Tocco, 1962). However, in previous studies with vertebrate guts (Schanker and Tocco, 1960, 1962), thymine and uracil were always transported by the same "carrier".

The "carrier" for thymine active transport did not appear to be completely saturated by the concentrations which were used. Active transport was observed over a concentration range of 0.156-15.60 nM/ml.

The presence of an active transport mechanism for the absorption of purines by the gut of any animal has not been

reported previously. In the rat, data have been obtained which showed that the purines, xanthine and hypoxanthine, were actively transported (secreted) into the lumen of the intestine (Berlin and Hawkins, 1968a, b). These observations were quite different from the results of the present studies which indicated that hypoxanthine and guanine were actively absorbed from the lumen of the chiton gut.

The possibility of two "carriers" was also indicated in the active transport of these two purines. The difference from the active transport mechanism for pyrimidines and that for purines was that guanine and hypoxanthine were both actively transported in the posterior regions of the gut, and only guanine was actively transported in the anterior gut.

Adenine and uric acid were not actively transported in these experiments. This observation was in agreement with the results of rat studies conducted by Berlin and Hawkins (1968a, b), and Oh, Dossetor, and Beck (1967).

The decrease in the rate of active transport of guanine in the anterior gut after return to aerobic conditions indicates that the "carrier" for guanine in the anterior gut was rendered ineffective after 2½ hours of anaerobic conditions. Since this was the only nitrogenous base which was unable to attain the previous S/M ratio or net serosal accumulation value suggested that the "carrier" for guanine may be different from that of other "carrier(s)" present. The sensi-

tivity of the hypoxanthine "carrier" to DNP was further proof that this "carrier" was truly dependent on a metabolic energy supply. However, the transport mechanism is not as sensitive as the active transport mechanisms for purines and pyrimidines in the mammalian gut (Schanker and Tocco, 1962, Berlin and Hawkins, 1968a, b).

It is of interest to speculate as to the significance of active transport system(s) for nitrogenous bases in the chiton gut. The absorbed nitrogenous bases in the chiton might be utilized in nucleic acid synthesis. It may be that the "carrier(s)" are homologous, or that they are really analogous active transport systems with differing phylogenetic origins. Whether or not a purine or pyrimidine active transport system(s) occur in guts of other invertebrate species is not known. Also, no active transport mechanism has been ascribed to occur in a gut which is lined with ciliated columnar epithelial cells (Fretter, 1937). In the mammalian system, the active transport mechanisms in the gut have all occurred in columnar epithelial cells.

The data from these studies and from earlier studies on the absorption of monosaccharides (Lawrence and Lawrence, 1967), amino acids (Greer and Lawrence, 1967), and inorganic ions (Lawrence and Mailman, 1967) in the gut of the chiton, indicate that this gut possesses a highly-developed active transport capability. This study is further evidence that

the gut of the chiton is indeed a very important organ for the absorption of necessary nutrients, and not only for formation of the fecal pellet as reported by Fretter (1937) and van Weel (1961).

SUMMARY

The everted sac technique of Crane and Wilson as modified by Lawrence was utilized to study the absorption of thymine, uracil, cytosine, guanine, hypoxanthine, adenine, and uric acid by the gut of the primitive mollusc Cryptochiton stelleri.

Thymine was actively transported in a concentration range of 0.156-15.60 nM/ml. Saturation kinetics were not observed at the highest concentration of thymine used. Thymine and guanine were actively transported in all regions of the chiton gut tested. Uracil was actively transported only in the proximal anterior gut, whereas hypoxanthine was actively transported only in the posterior gut regions. Cytosine, adenine, and uric acid displayed only fractional S/M ratios, and negative net serosal accumulation values which indicated that these nitrogenous bases were not actively transported in the chiton gut.

Studies using anaerobic conditions and DNP indicated that the active transport mechanism for the nitrogenous bases in the chiton gut were dependent on a constant supply of aerobic metabolic energy.

The active transport of the nitrogenous bases by the gut of the chiton displayed the following similarities to the mammalian active transport mechanisms:

1. Thymine, uracil, guanine, and hypoxanthine are moved

against their apparent concentration gradients.

2. Active transport of the above nitrogenous bases is dependent on an aerobic metabolic energy supply.

3. Purines and pyrimidines are not metabolized as they are absorbed.

4. Specificity is displayed in different regions of the gut of the chiton and for different purines and pyrimidines.

The following differences were observed in the active transport of the nitrogenous bases in the chiton from active transport displayed in the mammalian gut;

1. Hypoxanthine and guanine are actively transported in the chiton gut.

2. The active transport of hypoxanthine is less sensitive to DNP in the chiton.

3. The gut is capable of utilizing its active transport mechanism after prolonged anaerobic conditions.

4. Larger S/M ratios are developed with the active transport mechanism in the gut of the chiton.

5. The active transport of the nitrogenous bases occur across ciliated columnar epithelial cells.

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