

MEMBRANE TRANSPORT OF PHOSPHATE BY *HYMENOLEPIS DIMINUTA*

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ABSTRACT

The absorption of phosphate ion by *Hymenolepis diminuta* involved diffusion at all substrate concentrations tested. Mediated transport predominated at lower substrate concentrations and it exhibited saturation kinetics with V_{\max} and K_m values of 0.056 μmol ethanol-extractable inorganic phosphate/g ethanol extracted dry wt/2 min and 1.19 mM, respectively, obtained from the Lineweaver-Burk transformation. The V_{\max} and K_m values determined by the Hanes-Woolf plot were 0.136 μmol ethanol-extractable inorganic phosphate/g ethanol extracted dry wt/2 min and 3.64 mM, respectively. Absorption was influenced by changes in pH, temperature, and sodium ion concentration. Stable inorganic phosphate inhibited transport competitively. Various organic phosphates and common metabolic poisons introduced had no effect on transport. However, transport was inhibited by molybdate. From these data, it can be deduced that a separate transport system exists for phosphorus in *H. diminuta*.

INTRODUCTION

Intestinal parasites acquire inorganic substances largely, but not exclusively, from the intestinal contents of their hosts (von Brand, 1973). Read (1950) showed that *Hymenolepis diminuta* accumulated appreciable amounts of ^{32}P *in vivo* only when the labelled inorganic phosphate (Pi) was given orally to the host under conditions ensuring that it reached the worm before being absorbed by the host tissues. However, a slow phosphate accumulation also occurred when it was administered to the host (rats) intraperitoneally (Read, 1950). Inorganic phosphate absorbed by parasites is generally incorporated rapidly into various organic compounds (Lesuk and Anderson, 1940; Scheibel *et al.*, 1968; von Brand *et al.*, 1969). Von Brand (1973) stated that there was reason to assume that several inorganic substances, including Pi, entered cestodes by diffusion since Daugherty (1957) discovered that the Q_{10} of ^{32}P uptake by *H. diminuta* was very low. However, the authors were unable to find such information in the original article cited (Daugherty, 1957). Parasites usually grow at a much faster rate than free-living organisms, indicating that a large amount of Pi would be incorporated into phospholipids during membrane formation. Moreover, parasitic worms, like *H. diminuta*, produce enormous amounts of eggs containing many phosphorylated compounds. Hence, it would be inefficient for the growth and development of the parasite if it was to obtain such an important ion through diffusion. Therefore, the present investigation was undertaken to further elucidate the nature

of the transport mechanism involved in phosphate absorption in the rat tapeworm, *H. diminuta*.

MATERIALS AND METHODS

Hymenolepis diminuta was obtained from Carolina Biological Supply Co. (Burlington, North Carolina) as cysticercoids in adult *Tenebrio* sp. Male rats weighing 100 to 125 g were infected with 30 cysticercoids. Before and after infection, the rats were provided with water and food *ad lib*. Worms were flushed from the excised gut 10 days postinfection with Krebs-Ringer saline (KRT) containing 120 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl₂, 1.2 mM MgSO₄, and 25 mM tris (hydroxymethyl)-aminomethane-maleate buffered at pH 7.4 (Read *et al.*, 1963). For all experiments where the ionic product of calcium phosphate can easily exceed its solubility product, calcium was deleted from the KRT buffer used for preincubation and incubation.

Identification of the ethanol-extractable radio-phosphate

Experiments examined the nature of the ethanol-extractable inorganic phosphate after a two-minute incubation period. Samples of 0.5 g worms were preincubated in KRT buffer, followed by two minutes incubation in 0.2 mM labelled phosphate media. Upon removal from the incubation medium, the sample was rinsed rapidly in three changes of KRT buffer, blotted dry, and homogenized in six volumes of cold ethanol (80%). It was then centrifuged and the precipitate removed. To the supernatant fluid, 0.05 ml of 25% barium acetate was added to every milligram of inorganic phosphate in solution and the pH brought to 8.3 with sodium hydroxide. The resulting mixture was chilled and the precipitated phosphates collected by centrifugation, washed once with 70% ethanol, dried, and made into thick pastes with water. Dowex 50X8-200 (H⁺) was added until dissolution was complete. The supernatant fluid was separated from the resin, adjusted to pH 4.5 with 5 N sodium hydroxide, reduced to a small volume of 25 μ l, and used for paper chromatography and high voltage paper electrophoresis. The solvent system for paper chromatography was methyl cellosolve, pyridine, glacial acetic acid, and water (8:4:4:1), while 0.2 M borate buffer at pH 9.5 was used for high voltage paper electrophoresis. Electrophoresis was performed at 20V per centimeter for 45 min. Radioactive spots were located with a Berthold Beta Camera LB 292 (Berthold, Germany) and further determined by liquid scintillation counting of 0.5 cm strips of the processed paper.

Incubation studies

Standard incubation conditions were two minutes at 37°C in a water bath shaking at 100 oscillations per minute. All samples were incubated in radioactive monosodium phosphate adjusted within a range of 0.04 to 1.50 μ Ci/ μ mol depending on the nature of the experiment. For the concentration study (0.1 to 3.0 mM), a constant amount of ³H-polyethylene glycol (PEG 4000, NEN) of 1.39 μ Ci was also introduced into the incubation media adjusted to 0.3 μ Ci/ml. For all the other experiments, radioactive PEG was deleted from the incubation medium. Upon removal from the incubation medium the worms were rinsed rapidly in three changes of KRT buffer, blotted free of excess moisture on filter paper, and extracted overnight in tubes containing two milliliters of 70% ethanol. During the subsequent 24-h extraction, the tubes were agitated several times and one milliliter aliquots of the ethanol extracts were mixed with five milliliters of Biofluor (NEN) and radioactivity assayed using a Packard Tri-Carb 300 liquid scintillation spectrometer. Dry weights of ethanol-

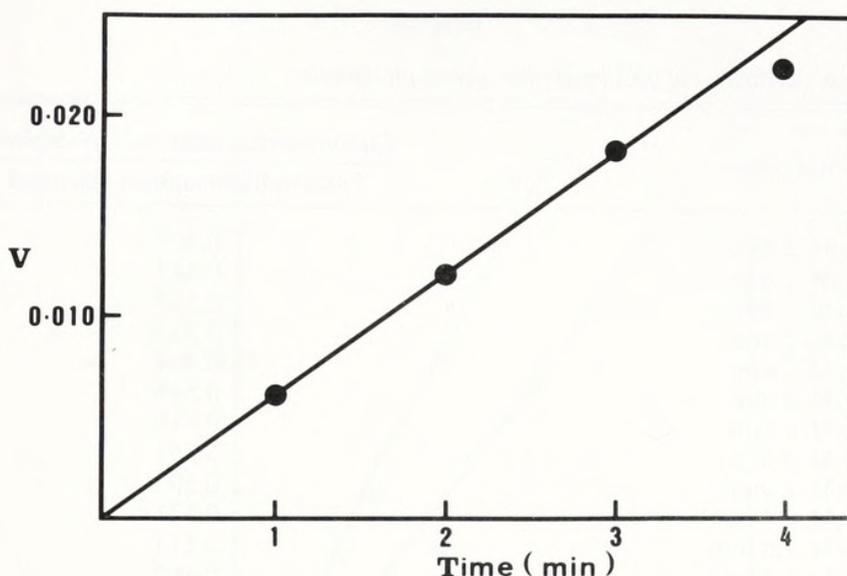


FIGURE 1. Absorption of 0.1 mM radiophosphate by *Hymenolepis diminuta* as a function of time (min). $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min. Each point is an average of three determinations. r^2 equals 0.87

extracted samples were obtained by heating worms at 110°C for five hours. Studies were also performed using balanced electrolyte solution (BES) containing: Na^+ 133 mM, K^+ 5 mM, Mg^{++} 1.2 mM, Cl^- 130 mM, HCO_3^- 10 mM, mannitol 25 mM, and equilibrated with 5% carbon dioxide in nitrogen to pH 7.0 (Podesta *et al.*, 1977b); and balanced saline solution (BSS) containing: NaCl 120 mM, KCl 5 mM, MgCl_2 , NaHCO_3 1 mM, 275 to 280 mosmol/kg, and equilibrated with 5% carbon dioxide in nitrogen to pH 7.4 (Ip and Fisher, 1982). Calcium and phosphate were deleted from the original formulae.

Efficiency of ethanol extraction

After ethanol extraction, worm samples were rinsed in 70% ethanol and digested in 0.4 ml of 60% perchloric acid and 0.8 ml of 30% hydrogen peroxide. The mixture was incubated at 70°C until colorless, cooled, and radioactivity assayed by liquid scintillation counting to determine the efficiency of extraction.

Monosodium of ^{32}P -phosphate and ^3H -polyethylene glycol were obtained from Amersham International plc (Amersham, United Kingdom). Reagent grade chemicals and stable phosphates were obtained from commercial sources.

Uptake velocities were expressed in micromoles of ethanol-extractable radioactive inorganic phosphate/g ethanol-extracted dry wt/2 min.

Student's *t* test was used to evaluate differences between means. Whenever applicable, linear regression was performed using the Apple II microcomputer.

RESULTS

The absorption rate of 0.1 mM radiophosphate by *H. diminuta* expressed as micromoles of ethanol-extractable phosphate per gram ethanol extracted dry weight was constant over a period of four minutes (Fig. 1). Therefore, two minutes was chosen as a suitable incubation time for all incubation studies.

TABLE I

Efficiency of ethanol extraction of absorbed radioactive phosphate

Conditions	Ethanol-extractable radiophosphate
	Total radiophosphate absorbed
0.1 mM, 2 min	0.608
0.3 mM, 2 min	0.542
0.5 mM, 2 min	0.513
0.7 mM, 2 min	0.533
1.0 mM, 2 min	0.464
1.5 mM, 2 min	0.548
2.0 mM, 2 min	0.519
2.5 mM, 2 min	0.561
3.0 mM, 2 min	0.509
0.1 mM, 0.5 min	0.521
0.1 mM, 1.0 min	0.513
0.1 mM, 2.0 min	0.489
0.1 mM, 10.0 min	0.539

Each value is an average from two experiments.

Identification of the ethanol-extractable radio-phosphate

Information on the identity of the ethanol-extractable phosphate was obtained by determining the ratio of radioactive inorganic phosphate to radioactive total phosphate in the ethanol extract. Results obtained by the various methods employed indicated that 95% of the extracted phosphate remained as the inorganic form.

Efficiency of ethanol extraction

Efficiency of radioactive phosphate extraction by ethanol from *H. diminuta* is presented in Table I. It was found to be consistently about 50% of the total amount of radiophosphate absorbed disregarding the length of the incubation period and the concentration of the external phosphate.

Effect of different media on phosphorus absorption

The absorptions of radiophosphate at various substrate concentrations tested in the BES and BSS were consistently lower than those in KRT (Table II).

TABLE II

Effect of different media on radiophosphate absorption in Hymenolepis diminuta

Radiophosphate concentration (mM)	Rates of uptake in different buffers (μ moles/g ethanol-extracted dry wt/2 min)		
	KRT*	BES**	BSS***
0.1	0.0071	0.0033	0.0045
0.3	0.0201	0.0118	0.0107
0.5	0.0331	0.0127	0.0222
1.0	0.0547	0.0179	0.0308
2.0	0.0718	0.0217	0.0660

* Krebs-Ringer tris-maleate buffer (Read *et al.*, 1963).

** Balanced electrolyte solution (Podesta *et al.*, 1977b).

*** Balanced saline solution (Ip and Fisher, 1982).

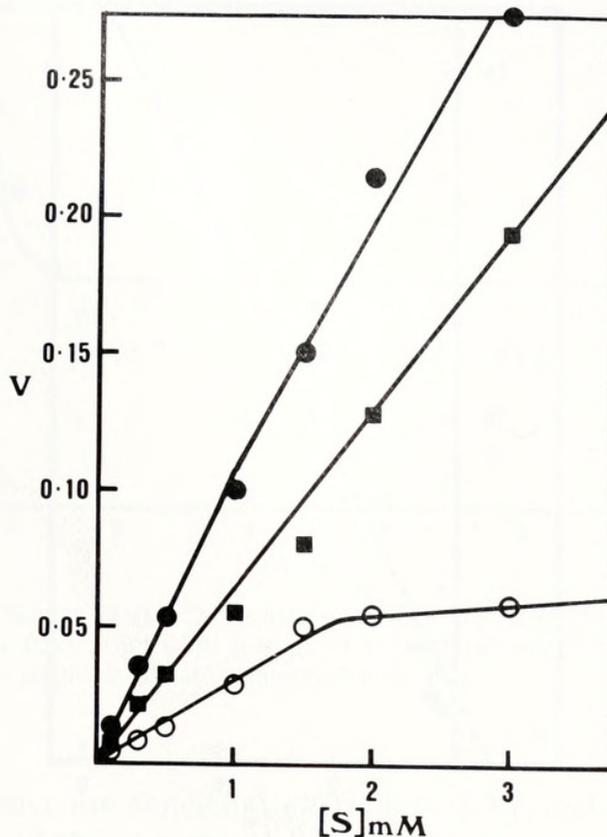


FIGURE 2. Absorption of radiophosphate by *Hymenolepis diminuta* as a function of phosphate concentration (0.01–3 mM). Each point is an average value from two experiments. S = phosphate concentration in mM; V = $\mu\text{mol/g}$ ethanol-extracted dry wt/2 min. ● = Total uptake; ○ = mediated component; ■ = diffusive component.

Effect of substrate concentration

The absorption process was a straight line relationship over a substrate concentration range of 0.03 mM to 20 mM suggesting diffusion might be involved. At lower substrate concentrations (0.01–3 mM) uptake was nonlinear and appeared to be a combination of mediated uptake and diffusion (Fig. 2). Subtracting the diffusive component obtained by deleting sodium from the incubation medium from the overall rate, the mediated process was unveiled with calculated V_{max} and K_m values of 0.056 μmol ethanol-extractable radioactive inorganic phosphate/g ethanol-extracted dry wt/2 min and 1.19 mM, respectively (Figs. 3, 4), obtained from the Lineweaver-Burk plot. Using Hanes-Woolf plot, the corresponding values of 0.136 μmol ethanol-extractable radioactive inorganic phosphate/g ethanol-extracted dry wt/2 min and 3.64 mM were obtained. From studies with ^3H -PEG, it was found that after rinsing and blotting dry three times, only 0.5 to 0.6 μl of the incubation medium was carried over as unstirred layer per sample of worm (0.09–0.10 g ethanol-extracted dry weight). Since this was only a minute volume and it did not significantly affect the calculated results, the unstirred layer was not labelled in the subsequent incubation studies. With increasing concentrations of stable phosphate in the incubation medium, the initial rate of uptake of 0.5 mM labelled phosphate decreased and then levelled off (Fig. 5). This result supported the hypothesis that mediated transport was involved and further demonstrated a component of uptake that was not inhibited by increases in stable phosphate concentrations.

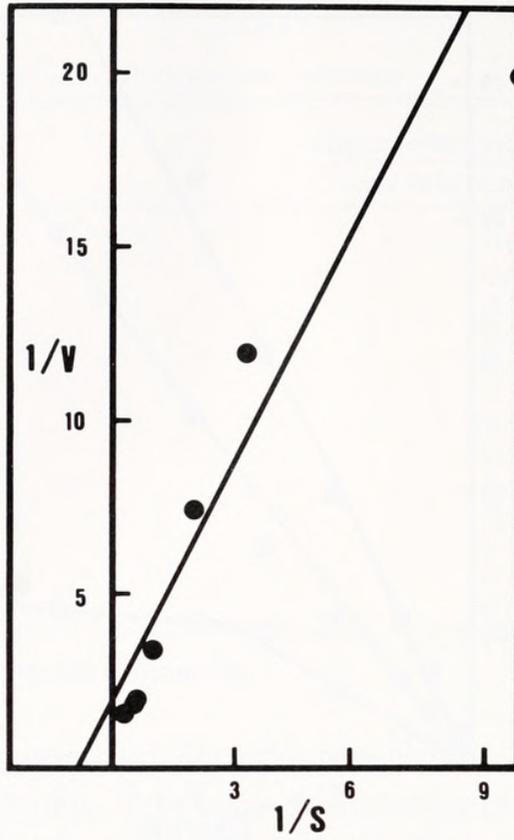


FIGURE 3. Lineweaver-Burk plot of radiophosphate absorption by *Hymenolepis diminuta*. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min; $S = \text{phosphate concentration in mM}$. $r^2 = 0.96$.

Effect of Na^+

Worms were preincubated in various KRT media deficient in sodium, potassium, calcium, magnesium, chloride, nitrate, and sulfate ions. They were rinsed in three

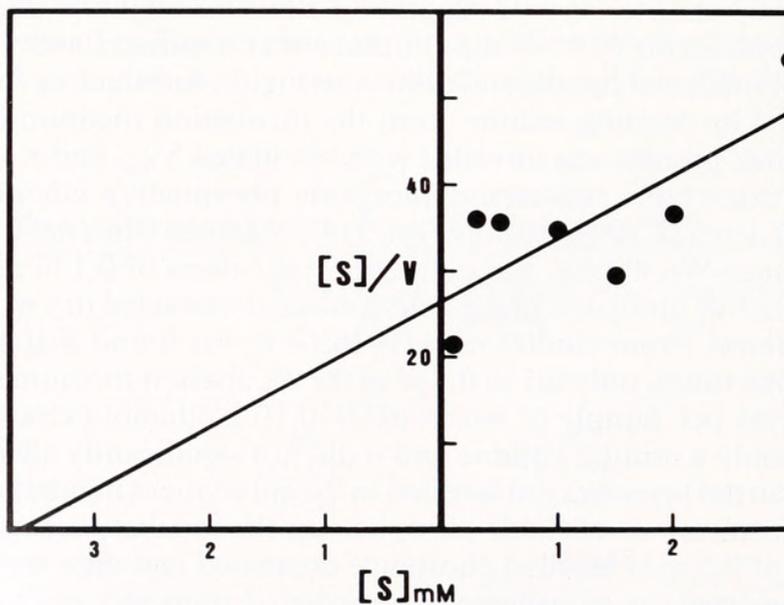


FIGURE 4. Hanes-Woolf plot of radiophosphate absorption by *Hymenolepis diminuta*. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min; $S = \text{substrate concentration in mM}$; $r^2 = 0.61$.

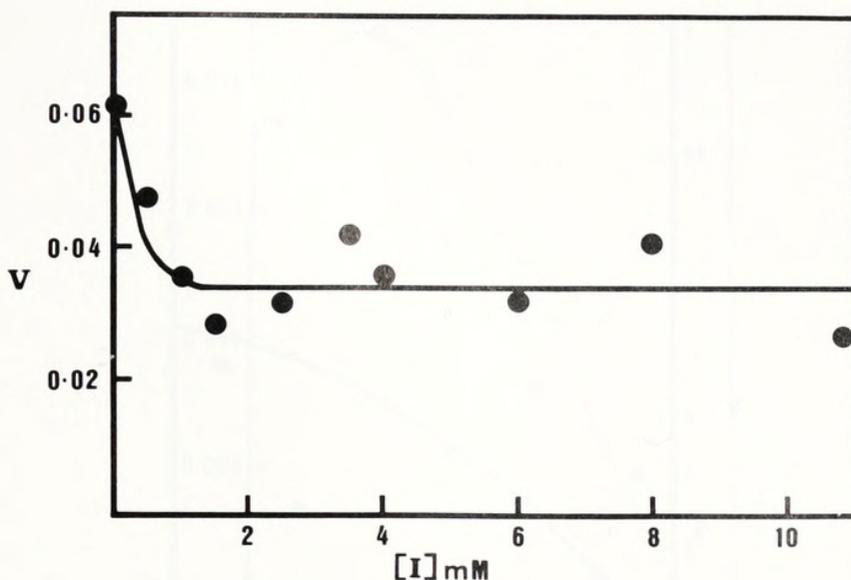


FIGURE 5. Effect of increasing stable phosphate concentration on uptake of 0.5 mM radiophosphate by *Hymenolepis diminuta*. Each point is an average value from two experiments. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min; $I = \text{stable phosphate concentration in mM}$.

changes of the respective ion deficient buffer, blotted dry, and then incubated in 0.1 mM radiophosphate in the absence of the respective ions. Absorption of labelled phosphate was affected by Na^+ but not by the other ions tested. Therefore, worms were incubated in 0.1 mM phosphate with various sodium ion concentrations (using choline as a replacement for Na^+). Mediated phosphate uptake by *H. diminuta* was sodium-dependent and a hyperbolic function of Na^+ concentration of the ambient medium (Fig. 6).

Effect of glucose and methionine

The absorption of 0.1 mM radiophosphate was not affected by the presence of either 1 mM glucose or 1 mM methionine in the incubation medium. The rates of absorption in the presence of glucose and methionine were 0.016 ± 0.002 ($n = 5$) and 0.018 ± 0.002 ($n = 5$) μmol ethanol-extractable Pi/g ethanol-extracted dry wt/2 min respectively, which were not significantly different from the control value of 0.019 ± 0.003 ($n = 5$, $P > 0.01$).

Effect of organic phosphates

Fructose-6-phosphate (F6P), glucose-1-phosphate (G1P), guanosine triphosphate (GTP), uridine diphosphate (UDP), NADP, and ATP (concentration 10 mM) were used to determine the specificity of the transport system. Absorption of 0.1 mM radiophosphate was not affected by NADP and ATP but was inhibited by F6P, G1P, GTP, and UDP. However, when the worms were incubated in the latter organic phosphate esters together with ATP, no signs of inhibition were observed (Table III).

Effect of metabolic poisons and molybdate

Sodium fluoride, potassium cyanide, parachloromercuribenzoic acid, iodoacetate, iodoacetamide, ouabain, dinitrophenol, and phlorizin were examined to determine

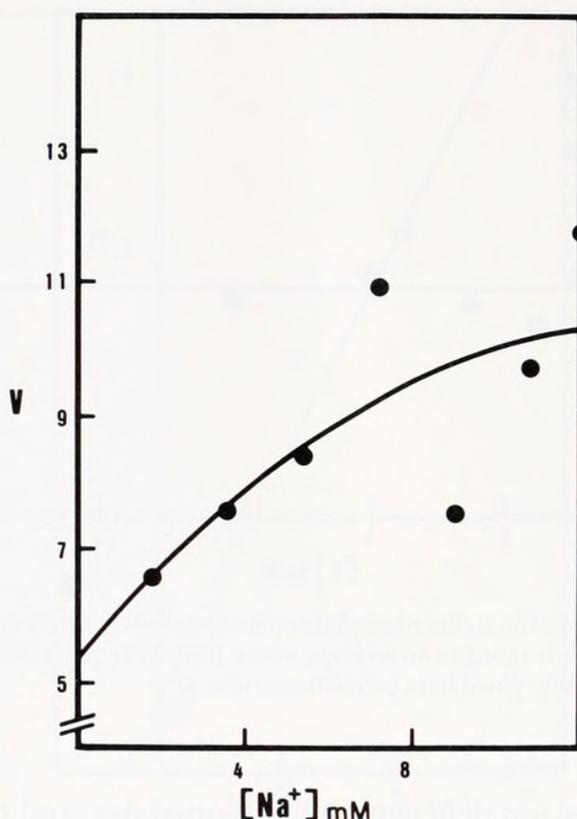


FIGURE 6. Effect of Na^+ concentration on mediated absorption of 0.1 mM radiophosphate by *Hymenolepis diminuta*. Each point is an average value from two experiments. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min.

their effects on 0.1 mM labelled phosphate absorption. None of the above poisons had any effect on the uptake of phosphate by *H. diminuta* when they were added to both the preincubation and incubation media. Molybdate, however, inhibited the mediated uptake of radiophosphate by 72% when compared to the control value.

Effect of pH and temperature

To study the effect of pH on the uptake of phosphate by *H. diminuta*, the worms were preincubated in KRT buffer at pH 7.4 and incubated for two minutes in 0.1

TABLE III

Effect of various organic phosphates on the uptake of 0.1 mM radiophosphates by Hymenolepis diminuta

Inhibitor(s) (10 mM)	% Inhibition of mediated process
F6P	30
G1P	28
GTP	23
UDP	41
NADP	0
ATP	0
F6P + ATP	0
G1P + ATP	0
GTP + ATP	0
UDP + ATP	0

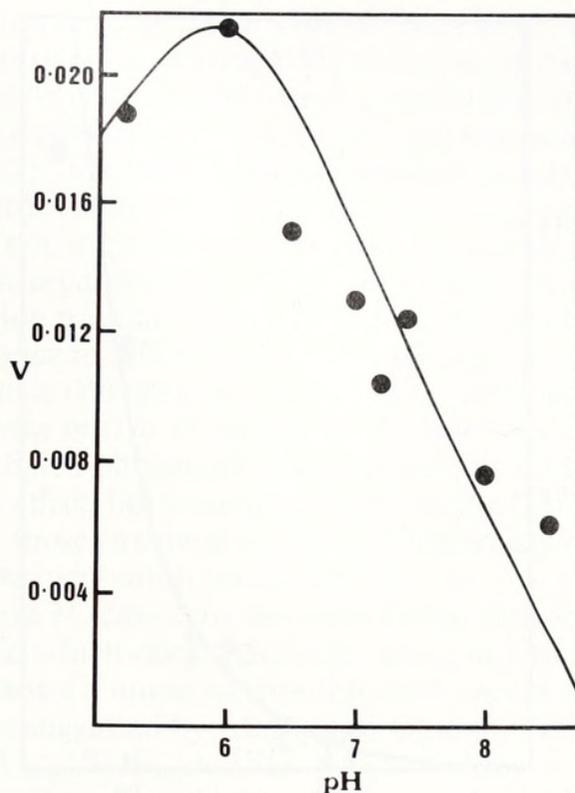


FIGURE 7. Effect of pH on the rate of 0.1 mM radiophosphate absorption by *H. diminuta*. Each point is an average value from two experiments. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min.

mM radiophosphate at various pHs (5.2 to 8.5) (Fig. 7). The velocity of the absorptive process increased rapidly and peaked at pH 6. The effect of temperature on phosphate uptake was determined by incubating the worms in 0.1 mM radiophosphate in the presence or absence of external sodium at various temperatures. Incubation in Na^+ yielded results for both the diffusive and mediated processes (Fig. 8). However, incubation in Na^+ deficient KRT buffer abolished the mediated process, producing results only for the linear component. Hence, the difference between the two velocities of the uptake expressed results of temperature effect on the mediated process. Absorption of phosphate was optimal at 37°C (Fig. 9).

DISCUSSION

The results of the short interval incubation experiments showed that 10-day-old *Hymenolepis diminuta* could absorb inorganic phosphate (P_i) from its surroundings. Ethanol could consistently extract only 50% of the radioactive phosphate absorbed (Table I) disregarding the incubation conditions. Even for an incubation period as short as 30 s, a similar large proportion of the absorbed phosphate was incorporated into the calcareous corpuscles and nucleic acids of the parasite (Ip, unpub. results). Since the time course study indicated that the free pool of inorganic phosphate within the worms, which was ethanol-extractable, did not attain a steady state relationship with the other internal phosphate pools and the external radioactive phosphate within four minutes of incubation indicating condition of initial uptake velocity (Table I, Fig. 1), the authors expressed the absorption rate in terms of micromoles of ethanol extractable radioactive phosphate per gram ethanol extracted dry weight per two minutes. More than 95% of the ethanol-extractable phosphate remained unmetabolized in the worm tissues.

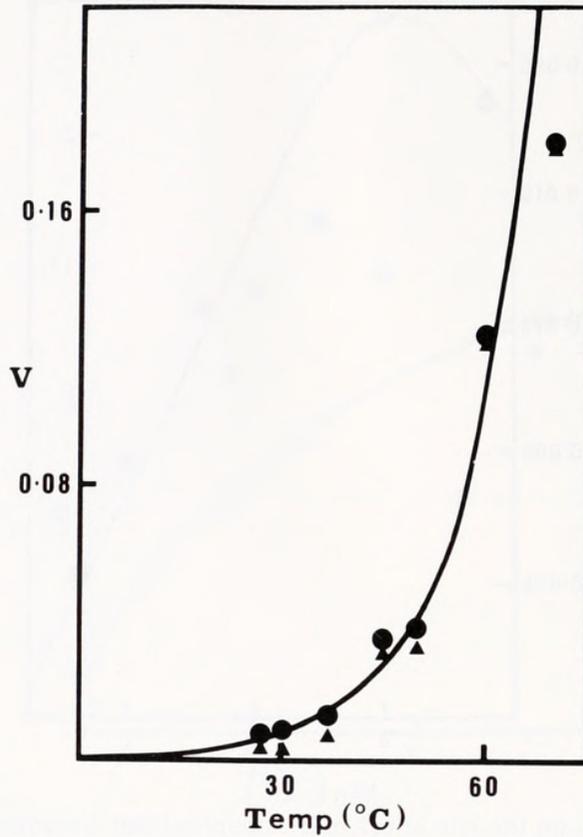


FIGURE 8. Effect of temperature on rate of total absorption and Na-independent uptake of 0.1 mM radiophosphate by *Hymenolepis diminuta*. Each point is an average value from two experiments. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min. ● = Total uptake; ▲ = Na-independent uptake.

Krebs-Ringer tris-maleate (KRT) buffer was used in all incubation studies because of its ubiquity in other membrane transport studies of *H. diminuta*. It has been proposed that maleate in KRT may affect transport processes (Roth *et al.*, 1976). However, there are no clear indications that maleate specifically inhibits any trans-

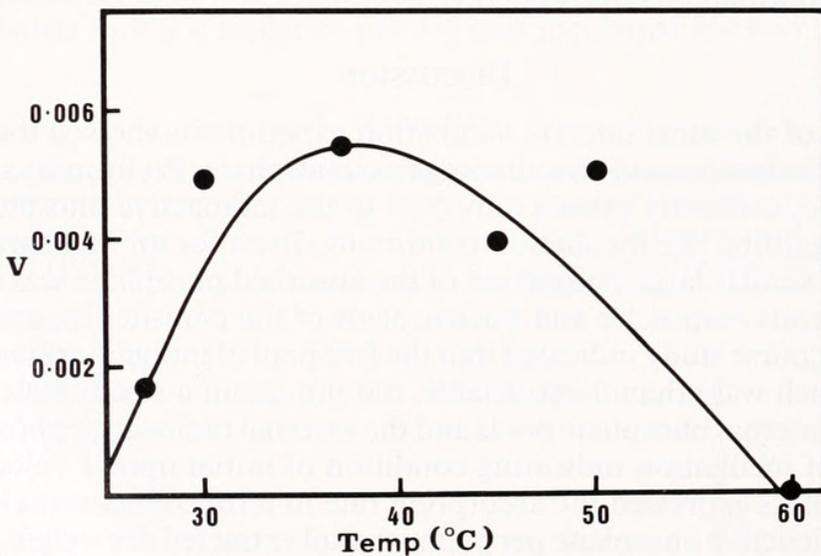


FIGURE 9. Effect of temperature on rate of mediated uptake of 0.1 mM of radiophosphate by *Hymenolepis diminuta*. $V = \mu\text{mol/g}$ ethanol-extracted dry wt/2 min.

port process in *H. diminuta*, although Podesta *et al.* (1977b) reported an apparent reduction in the influx of glucose using KRT as the incubation media. Furthermore, the uptake rates of phosphate in KRT were considerably higher than those of balanced electrolyte solution (Podesta *et al.*, 1977b) and balanced saline solution (Ip and Fisher, 1982) (Table I). The adsorption of radioactive substance to the 'unstirred layer' of the brush border membrane of *H. diminuta* was minimum after rinsing and blot-drying, and did not impose serious errors on analyses of results for phosphate absorption. There was argument that the 'unstirred layer' would contribute errors to transport determination not only due to the adsorption phenomenon but also to the requirement of substance to diffuse across such barriers before becoming available to the transport mechanism (Podesta, 1977). Since the 'unstirred layer' is naturally present in the gut of the host *in vivo*, it was not the authors' intention to report V_{\max} and K_m values of the mediated phosphate transport process under an artificial situation of no 'unstirred layer' effect, but to demonstrate a distinct phosphate transport mechanism different from those involved in other transport phenomena in *H. diminuta* under standard *in vitro* incubation conditions.

Phosphate uptake in *H. diminuta* consisted of two components: A saturable, Na^+ dependent component which obeys Michaelis-Menton kinetics and a Na^+ independent process which shows a linear relationship with increasing extracellular Pi concentration (Fig. 2). As suggested by Atkins and Gardner (1977), direct fit of the two term function $v = (V_{\max}\{S\}/K_m + \{S\}) + k\{S\}$ (where v = micromoles ethanol-extractable radioactive Pi/g ethanol/extracted dry wt/2 min, V_{\max} = maximum velocity, K_m = Michaelis-Menten constant, and k = the rate constant of the linear component) to the data obtained from concentration studies was performed. Subtraction of the rate of uptake of the Na^+ independent component (diffusion) from the overall rate yielded a corrected rate for the saturable process with V_{\max} and K_m values of $0.056 \mu\text{mol}$ ethanol-extractable radioactive Pi/g ethanol-extracted dry wt/2 min and 1.19 mM , respectively, as obtained from the Lineweaver-Burk plot and corresponding values of $0.136 \mu\text{mol}$ ethanol-extractable radioactive Pi/g ethanol extracted dry wt/2 min and 3.64 mM as determined by the Hanes-Woolf plot. Such a discrepancy can be due to the inaccuracy of the Lineweaver-Burk determination of kinetic constants as there are relatively few points at the high end of the $1/(S)$ scale, and it is these points that are most heavily weighted in such determinations. Moreover, small errors in the determination of V_{\max} are magnified when reciprocals are taken. The results of the present investigation contradict previous suggestion that absorption of Pi was due to diffusion (von Brand, 1973). Although diffusion occurred at all substrate concentrations tested, mediated transport predominated at substrate concentrations below 1.5 mM (Fig. 2). Mediated phosphate absorption was affected by the concentration of Na^+ in the ambient medium. This finding is consistent with that reported on the transport of inorganic phosphate transport by SV3T3 cultured cells (Brown and Lamb, 1975).

The cations and anions K^+ , Ca^{++} , Mg^{++} , Cl^- , SO_4^{--} , and NO_3^- had no effect on transport activity. The sodium requirement, as demonstrated in numerous other systems (Read *et al.*, 1974; Schultz *et al.*, 1974; Crane, 1977), suggests that sodium and phosphate may be cotransported by a catalytic protein 'carrier' system in *H. diminuta* similar to that proposed by Crane (1965) for glucose transport in mammalian intestine. Metabolic poisons, in particular ouabain, had no inhibitory effect on the rate of phosphate absorption in *H. diminuta*. Such a Na^+ -coupled active transport system usually requires an ouabain-sensitive Na^+ - K^+ activated ATPase (Schultz *et al.*, 1974; Crane, 1977). The observation that ouabain has no effect on glucose transport in *H. diminuta* (Lee *et al.*, 1963; Dike and Read, 1971b) raises doubt to the

presence of such an enzyme in this parasite and its involvement in phosphate transport in the present study. However, *H. diminuta* is impermeable to ouabain (Gallogly, 1972) and the ouabain-sensitive $\text{Na}^+\text{-K}^+$ activated ATPase may not be localized in the surface membrane(s) of the brush border. Recently, the Na^+ extrusion mechanism was found to be localized below the tegument (Podesta *et al.*, 1977a) and this hypothesis is sustained by Lumsden and Murphy (1980). As such, the absence of any effect of ouabain on phosphate uptake in *H. diminuta* is expected. The mediated transport of phosphate was inhibited by molybdate, possibly the result of structural damage of the surface catalytic protein carrier molecules.

Arme and Read (1970) and Dike and Read (1971a) showed that phosphatase activity on the surface membrane of *H. diminuta* was totally inhibited by ATP. The absence of inhibitory effect when ATP was added to the incubation media in the presence of either F6P, G1P, GTP, or UDP indicated that these organic phosphates had no direct effect on inorganic phosphate absorption. The apparent inhibitory effect of these organic phosphates was a result of increasing inorganic phosphate concentration in the external media after hydrolysis of the respective phosphate esters by the surface enzyme. Since phosphate transport was not affected by a variety of organic phosphate esters, glucose, and methionine, it may be concluded that phosphate transport in *H. diminuta* occurs through a different mechanism with high substrate specificity.

The absorption of phosphate by the worm was sensitive to temperature and pH of the ambient medium. Optimal absorption occurred at 37°C. At lower temperatures, the surface enzymes required for mediated phosphate uptake are probably inactivated and at higher temperatures, the decreased rate is likely due to damage of the cestode integument. It is significant that maximum absorption occurred at pH 6 at which the major inorganic phosphate exists as H_2PO_4^- in solution. Hence, diffusion can play a major part in phosphate uptake since this phosphate is less negatively charged as compared to the other possible phosphate ions.

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