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The Kinetic and Genetic Isolation of Two Histidine Transport-mediating Systems.

by

Clare Keating Woodward

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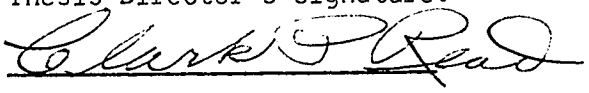
Thesis Director's signature:



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INTRODUCTION

General: A large body of evidence has accumulated indicating that in living cells the transport of amino acids across membranes is mediated through specific sites or carrier systems. The number, location, structure and mechanism of action of qualitatively different sites or carriers may thus be expressed in the kinetic characteristics and in the genetic control of transport systems. Historically, research on amino acid uptake by cells can be divided into these two general categories: 1) Studies on the kinetics of amino acid uptake, including the inhibitory effects of other amino acids, have demonstrated the phenomenon of mediation and led to hypotheses of qualitatively different carriers. The delineation of the amino acid transport loci of several different membranes has been approached mainly by kinetic analyses of inhibition among the amino acids, the various systems being parasitic flatworms (26-30), mammalian tumor cells (9, 24), mammalian erythrocytes (8), vertebrate intestine (40), rat diaphram (5), vertebrate pancreas slices (3), fungi (11, 12, 22, 31, 35), and bacteria (17). 2) Genetic studies have been concerned with the inheritance of alterations in uptake phenotypes. Genetic analyses have been restricted to microorganisms (35, 17). Several comprehensive reviews of the transport field are available (6, 7, 20).

Amino Acid Inhibition Studies: The basic and the neutral amino acids comprise separate transport families in all tissues in which amino acid transport has been studied. The members of an amino acid transport family enter a cell through a transport-mediating system which is inhibited by amino acids of the same family, but not by amino acids of another family. Read and coworkers have demonstrated in Hymenolepis diminuta and in several other tissues that the neutral and the basic amino acids form two separate transport groups.

Amino acid uptake is more complex than can be explained by simple diffusion. Although there is evidence that carriers located on the membrane facilitate uptake, it is not clear whether binding sites are different from carriers.

Using Ehrlich ascites tumor cells, Christenson (10) has concluded that there are at least seven "discrete transport-mediating systems." He is of the opinion that the transport-mediating system of one amino acid is chemically and spatially separate from that for a second amino acid, based on the demonstration of differences in the relative inhibition patterns produced by several amino acids with each of two test amino acids. Thus, an "alanine-preferring" system is differentiated from a "leucine-preferring" system by two main lines of evidence: 1) alanine inhibits the uptake of glycine more than it does the uptake of leucine and valine, whereas leucine inhibits the uptake of valine more than the uptake of alanine or glycine, and 2) glycine is accumulated by the cells to a greater extent than leucine and valine (24).

Read, Rothman and Simmons (29) demonstrated similar phenomena with the neutral amino acid groupings in Hymenolepis and interpreted the data to indicate at least two kinetically separate, but not necessarily spatially separate neutral amino acid systems.

Christenson also concludes that there are three separate transport-mediating loci for cationic amino acids, one which resembles the leucine-preferring system, one with which the neutral amino acids compete (10), and one with which the neutral amino acids do not compete (as judged by the observation that mediated entry is not completely inhibited by high concentrations of neutral amino acids) (10).

Christenson claims another separate transport-mediating system for α-α-diethyl glycine since, 1) it has a small inhibitory effect on other amino acids but is itself strongly inhibited by other amino acids, and 2) the K_i values of the inhibition of α-α-diethyl glycine uptake by these amino acids are not comparable to the K_m values of entry of these amino acids through other systems (10).

The general model of amino acid entry conceived by Christenson is as follows: Amino acid entry through membranes is facilitated by multiple transport-mediating loci, and each locus has a binding and transport affinity for several amino acids, albeit, a given locus will exhibit greater affinity for one amino acid than for another. He describes this phenomenon as multiple sites with overlapping affinities.

There are no data conclusively identifying the binding site with the transport site, and not everyone agrees that they are

identical. For example, Begin and Scholefield claim that, in mouse pancreas, L-valine and glycine are each transported by a separate system with no overlapping transport affinity with other systems since the K_m values did not agree with the K_i values. They did suggest, however, that there might exist overlapping binding (abortive transport) affinity with other systems (3). At least one investigator has concluded that the carrier molecule binds two molecules of amino acid before transport can occur. Working with Ehrlich ascites cells, Jacquez (18) found that the initial uptake rate of L-tryptophane is increased if specific amino acids are simultaneously present in the medium or if the cells are preloaded with one of these amino acids. In order to explain the stimulation in simultaneous incubation, he concluded that an amino acid:carrier ratio of 2:1 must obtain.

Kilejian (19), studying proline uptake and efflux in Hymenolepis diminuta concluded that both influx and efflux are mediated and that the uptake system possesses two saturable "sites," meaning, presumably, that there are at least two separate loci for the uptake of proline.

DeBusk and DeBusk (11) have studied the kinetics of the phenylalanine mediating system in conidia of wild type Neurospora. They observed that all of the naturally occurring amino acids inhibit phenylalanine uptake but that some inhibit to a greater extent than others. Methionine, leucine, tryptophane and tyrosine are effective inhibitors, alanine, serine and valine less so, histidine and glycine inhibit about fifty per cent, while arginine and lysine

are the least effective (20-40 per cent inhibition). They point out that these uptake inhibition patterns are the same as those obtained in growth inhibition of phenylalanine-requiring mutants of Neurospora (4) and thus the growth inhibition has its basis in transport rather than metabolic phenomena. That the inhibition patterns are the same in these two cases also shows that the phenylalanine requirement does not affect the wild type uptake mechanisms.

Roess and DeBusk (31) conducted similar experiments with the arginine-mediating system, using conidia of wild type Neurospora. In like manner, arginine uptake is inhibited by most amino acids but to varying degrees. Lysine was the most effective inhibitor; phenylalanine and histidine were intermediate; and proline was least effective. It has repeatedly been found in Neurospora that the growth of lysine-requiring mutants is inhibited by arginine (14), and that the growth of arginine-requiring mutants is inhibited by lysine (34). Using canavanine resistant mutants of Neurospora, Bauerle and Garner (2) showed that arginine inhibits lysine uptake and that lysine inhibits arginine uptake. Zalokar (46) has demonstrated that proline is accumulated by Neurospora mycelia against a concentration difference.

The uptake of amino acids by bacteria has been studied rather extensively, but no attempt will be made to review this work. Extensive reviews of this field have been completed by Holden (16) and Kepes and Cohen (17). Mention will be made, however, of the

studies by Ames (1) of histidine uptake in histidine-requiring mutants of Salmonella typhimurium since the situation seems to differ from that found here in Hymenolepis and Neurospora. Ames found that when histidine is in low concentration in the medium, no other amino acid(s) inhibits its uptake, but when in high concentration in the medium histidine uptake is inhibited by other amino acids. She concluded that two histidine entry loci exist in Salmonella, one specific and operative (observable) at low concentrations and one non-specific and observable only at high histidine concentrations.

The Genetic Control of Transport: Mathieson and Catcheside (22) demonstrated that the growth of certain histidine-requiring mutants of Neurospora is 1) partially reduced when one of the following amino acids is present in histidine-supplemented growth media: Arginine, methionine, lysine, tryptophane, glycine, phenylalanine, and leucine, and 2) completely inhibited when one of the following pairs of amino acids is present in the histidine-supplemented growth medium: Arginine + methionine, arginine + tryptophane, arginine + glycine, lysine + methionine, lysine + tryptophane, or lysine + glycine. They also showed that the degree of growth inhibition of a given histidine mutant by these amino acids is a function of the genetic background of the mutant, and that the effect of the inhibitors was to prevent the uptake of histidine from the growth medium. They showed that histidine was accumulated against a concentration difference by the mutants and that as growth proceeded the histidine gradually disappeared from the mycelia. The

addition of inhibitors following the accumulation of histidine had no effect upon its utilization as judged by growth. It was also shown by these authors that wild type Neurospora accumulates histidine from the growth medium and that the addition to the medium of arginine and methionine prevents accumulation. From this they concluded that the mutation causing the histidine requirement did not create the inhibition phenomenon but made it more easy to observe.

Stadler (35) has isolated and genetically mapped, at one locus, thirty mutants of Neurospora which are resistant to 4-methyl tryptophane (these mutants are called mtr mutants). Mycelial pads of six of these mutants were tested for tryptophane uptake and all showed a greatly reduced rate of tryptophane uptake compared to wild type. These mutants were also found to be deficient in their uptake of other amino acids, e.g., tyrosine and phenylalanine uptake were greatly reduced; methionine, valine and leucine uptake was reduced by approximately 75 per cent; histidine uptake was reduced about 50 per cent; arginine and lysine uptake was not affected by the mtr mutation. It appears that these mutants are missing a general aromatic amino acid-mediating system which also has some affinity for the neutral amino acids but not for the basic amino acids.

The generalization permissible with the studies to date is that the basic and the neutral amino acids comprise two kinetically separate transport-mediating systems. There is evidence for several kinetically separate neutral amino acid-transporting systems with what can be described as overlapping affinities.

Although it is often assumed, it has not been shown that kinetically separable transport-mediating systems are chemically and

spatially separate. It is possible that a single carrier molecule has several qualitatively different attachment sites which may interact in any of the following ways: 1) binding at one site does not affect subsequent binding at a second site, and vice versa. 2) binding at one site may increase or decrease subsequent binding at a second site, whereas binding at the second site does not affect the first, 3) binding at one site affects binding at the other, and vice versa. It is also possible that a single carrier molecule with a single binding site exists in several forms which are in equilibrium with each other and each of which has a different rate of binding for a given substrate, and possibly different rates of mobilization across the membrane barrier. This equilibrium could be shifted either way by the appropriate amino acid.

The work to be described here shows that the amino acid histidine inhibits and is inhibited by arginine and neutral amino acids, whereas arginine neither inhibits nor is inhibited by neutral amino acids. This phenomenon was observed in the parasitic tapeworm, Hymenolepis diminuta using ^{14}C uptake as the measure of entry, and in Neurospora crassa using growth of histidine-requiring mutants. If histidine can be shown to be transported by two kinetically isolable transport-mediating systems, corresponding respectively to the neutral and the basic amino acid transport systems, then histidine uptake may be used as a tool to select mutants which lack one or the other of these kinetically isolable systems. Study of the genetics of these mutants may then yield insight into the chemical relationships underlying the amino acid family groupings found

to be characteristic of all amino acid transport systems. It was to these questions that the following experiments were addressed.

EXPERIMENTAL PROCEDURES

Hymenolepis diminuta

Husbandry: The procedures for maintaining and harvesting the tapeworm, Hymenolepis diminuta, have been standardized and described by Read, Rothman and Simmons (29). Their procedures were used in the present study and are summarized as follows: Male albino rats of a Sprague-Dawley strain (obtained from Höltzman Rat Co.) weighing about 130 gm were infected with 30 Hymenolepis cysticercoids collected from Tribolium confusum or Tenebrio molitor. During the period of worm growth the rats were housed in wire mesh cages in groups of 15 and were fed Purina Laboratory Chow. Worms were collected from the rats 10 days ± 3 hours after infection. At harvest the rats were killed by snapping their necks, the guts were excised and flushed at room temperature with Ringers solution buffered at pH 7.4 with 25 mM TRIS-hydroxymethylaminomethane-maleate (29), referred to hereafter as KRT. Following harvest, the worms were rinsed with KRT 3 times prior to use in the experiments.

Uptake Assay: Worms from 8 to 15 rats were randomized and divided into 5 worm samples, placed in 5 ml KRT and preincubated for 60 minutes at 37 C in a shaking water bath. Following this preincubation treatment, the worms were removed from KRT, blotted on filter paper and placed in 4 ml of appropriate incubation liquid where they were incubated for varying lengths of time at 37 C. After incubation

the worms were removed, rinsed 3 times in KRT, blotted on filter paper, placed in 2 ml 70 per cent ethanol, and extracted for 24 hours. The worms were removed, dried at 90 C for 24 hours and weighed. The ^{14}C extracted was measured by taking a 0.5 ml sample of the ethanol extract, plancheting in duplicate on 1.5 inch diameter stainless steel planchets, and counting (minimum of 5000 counts) on a Bechman Low Beta II gas flow counter. Calculations of uptake were corrected both for background count and for dilution by worm water (body water of the whole worms introduced into the ethanol). Since worm wet weight is approximately five times worm dry weight (100:19.2) (29), the total volume of the extract was 2 ml plus four times the dry weight of the worms extracted.

The uniformly-labelled L-histidine ^{14}C was obtained from New England Nuclear Corporation and the unlabelled amino acids were purchased from the California Corporation for Biochemical Research.

Uptake and standard deviations were determined on a 1620 IBM computer. The curves in figures 2, 4, 5 were plotted by the 7040 IBM computer for the best fit to a rectangular hyperbola by least squares analysis giving least weight to the points with larger standard deviations. Each data point is the average of at least 5 replications. Amino acid analyses were done with a Technicon Auto Analyzer fitted with a Packard Flow Scintillation Analyzer.

Neurospora crassa

Husbandry: The various mutants of Neurospora were maintained on basal agar medium (44) supplemented with 10 mg of the required

amino acid(s) per 100 ml medium. Mycelia used in the uptake studies were obtained by inoculating 5 day old conidia into basal liquid media supplemented with the appropriate amino acids plus 100 mg per cent yeast extract and incubating for 24 to 48 hours at 28 C in stationary culture.

Strains: The histidine-requiring mutants H3 (K34) and H6 were obtained from the Fungal Genetics Stock Center, Dartmouth College, Hanover, New Hampshire, and strains H3 (K26) and H3 (K12) were generously provided by Professor D. G. Catcheside of the Australian National University, Canberra, Australia. The mutants used in the genetic analyses were obtained from the Fungal Genetics Stock Center.

The Isolation of Uptake Mutants: Since the rationale for using Neurospora in these analyses of amino acid transport was based on the premise that transport through membranes is at least in part genetically determined, it is necessary to describe in some detail the criteria used to screen and select mutant strains altered in their amino acid uptake phenotype. The histidine-requiring mutants used in these studies are characterized by an absolute growth requirement for histidine. In the presence of histidine the mutants grow at the same rate as wild type, implying an unimpaired histidine transport mechanism. However, the same histidine mutants show reduced growth rates when either excess arginine or excess methionine is added to the histidine supplemented growth media and no growth at all when excess arginine and excess methionine are added. These facts imply that histidine is transported into the cell via two

"carriers," one a histidine-arginine carrier and the other a histidine-methionine carrier, and that the presence of excess arginine and/or methionine inhibits the passage of histidine through the membrane. The screening criterion used to select mutational alterations of histidine uptake was the inability of a strain to grow on histidine in the presence of arginine, in the one case, or in the presence of methionine in the other.

The histidine mutants were grown on minimal medium supplemented with histidine, conidia were harvested in distilled water, filtered through cheese cloth, and exposed to 25,000 r of X-irradiation at a concentration of 10^6 conidia per ml. Four ml of the irradiated suspension were transferred into 1) 200 ml liquid medium supplemented with 10 mg per cent histidine and 50 mg per cent arginine, and 2) 200 ml liquid medium with 10 mg per cent histidine and 50 mg per cent methionine. Wild type mycelia were filtered from the suspension over a period of two days after which the remaining conidia were plated onto minimal agar medium supplemented with 10 mg per cent histidine (43). From such plates 2,000 colonies were isolated into individual culture tubes and the resultant strains were tested for altered histidine uptake. Conidia from each strain were tested for growth on 1) minimal liquid, 2) minimal liquid plus 10 mg per cent histidine, 3) minimal liquid plus histidine plus 50 mg per cent arginine, 4) minimal liquid plus histidine plus 50 mg per cent methionine, and 5) minimal liquid plus the three amino acids. Strains showing a negative growth response on 1, 3 and 5, or 1, 4 and 5 were saved for further study.

Genetic Analyses: In order to determine whether the altered uptake of the isolated strains is attributable to genetic or to non-genetic phenomena, crosses were made between suspected transport mutants and at least two morphological mutants from each of the seven linkage groups (nuclei of Neurospora contain seven haploid chromosomes and genetic analyses have shown seven linkage groups). Fifteen morphological mutants representing widely spaced positions on each of the linkage groups were used as the female parents in the crosses. All crosses were made on corn meal agar, and the fertilized strains were incubated in the dark at room temperature for three weeks prior to the isolation of ascospores. The ascospores were isolated singly in separate test tubes containing agar medium supplemented with histidine, then activated for 30 minutes at 60 C. Conidia from the germinated progeny were then tested for phenotype. First, strains of marker type morphology were scored, then each was tested on 1) minimal liquid, and 2) minimal liquid plus 10 mg per cent histidine. Of these, the strains that grew in histidine but not in minimal medium were tested on 1) minimal liquid plus 10 mg per cent histidine plus 50 mg per cent arginine, 2) minimal liquid plus histidine plus 50 mg per cent methionine, and 3) minimal liquid plus the three amino acids. Those strains showing a negative growth response on 3 only were scored at wild type (tpt^+) genotype; those with negative growth response on 2 and 3 or on 1 and 3 were scored as tpt^- (transport altered) mutants.

Complementation Analyses: The complementarity of two mutant nuclei of Neurospora can be tested by forcing them to reside

in the same mycelium. These heterocaryons can be induced by placing conidia of nutritionally deficient strains onto unsupplemented medium where their incipient hyphae fuse, letting both types of nucleus into a common cytoplasm.

Conidia from each of the seven suspected mutants were harvested with distilled water and concentrated to more than 10^6 conidia per ml. With a Pasteur pipet, three drops from each suspension were spaced on seven agar plates. On a given plate, three drops from a different suspension were placed onto the same agar, two overlapping and one new spot, permitting on each plate a control of each of the two strains involved and two overlapping spots providing opportunity for heterocaryon formation (42).

Complementation tests were made in all pair-wise combinations of the seven mutants on 4 different media, 1) minimal plus 10 mg per cent histidine, 2) minimal plus 10 mg per cent histidine plus 50 mg per cent arginine, 3) minimal plus 10 mg per cent histidine plus 50 mg per cent methionine, and 4) minimal plus the three amino acids.

RESULTS AND DISCUSSION

In all cases where the uptake of amino acids has been studied it has been found that the neutral and the basic amino acids comprise two kinetically isolable uptake systems; that is, within each group there is mutual inhibition of uptake but little or no inhibition between groups. The work presented here provides evidence, 1) that, in Hymenolepis diminuta and Neurospora crassa, histidine uptake occurs through two kinetically separate uptake systems which correspond respectively to neutral and basic amino acid transport-mediating systems, and 2) that the genetic distinction studies with Neurospora point to the chemical separability of the two uptake systems.

1. Evidence that histidine uptake occurs through both neutral and basic amono acid transport-mediating systems:

A. Hymenolepis diminuta

i) Uptake: Tapeworms have certain advantages for the study of transport phenomena since all nutrients enter the organism through the external body surface. Considerable data are available to show that mediated transport of amino acids occurs in these animals (26-30, 19, 33). Read, Rothman and Simmons (29) found that in Hymenolepis the neutral amino acids are mutually inhibitory, as are the basic amino acids, but that there is no inhibition between the neutral and the basic amino acids (Table I). Since the inhibition studies by Read and coworkers indicate that in Hymenolepis

TABLE I

LACK OF RECIPROCAL INHIBITION OF ARGININE AND PHENYLALANINE TRANSPORT

Uptake of ^{14}C-labeled amino acid measured in 2 minute incubations. Values for incubation media are mM concentrations. Uptake expressed as micromoles ^{14}C-amino acid/gram dry weight/2 minutes.

Incubation medium*	Uptake
0.40 arginine-^{14}C	1.05 ± 0.11
0.40 arginine-^{14}C + 0.40 phenylalanine	1.03 ± 0.06
0.80 arginine-^{14}C	1.01 ± 0.06
0.80 arginine-^{14}C + 0.80 phenylalanine	1.08 ± 0.08
0.40 phenylalanine-^{14}C	1.47 ± 0.10
0.40 phenylalanine-^{14}C + 0.40 arginine	1.40 ± 0.12
0.80 phenylalanine-^{14}C	1.83 ± 0.19
0.80 phenylalanine-^{14}C + 0.80 arginine	1.70 ± 0.20

* Millimolar quantities

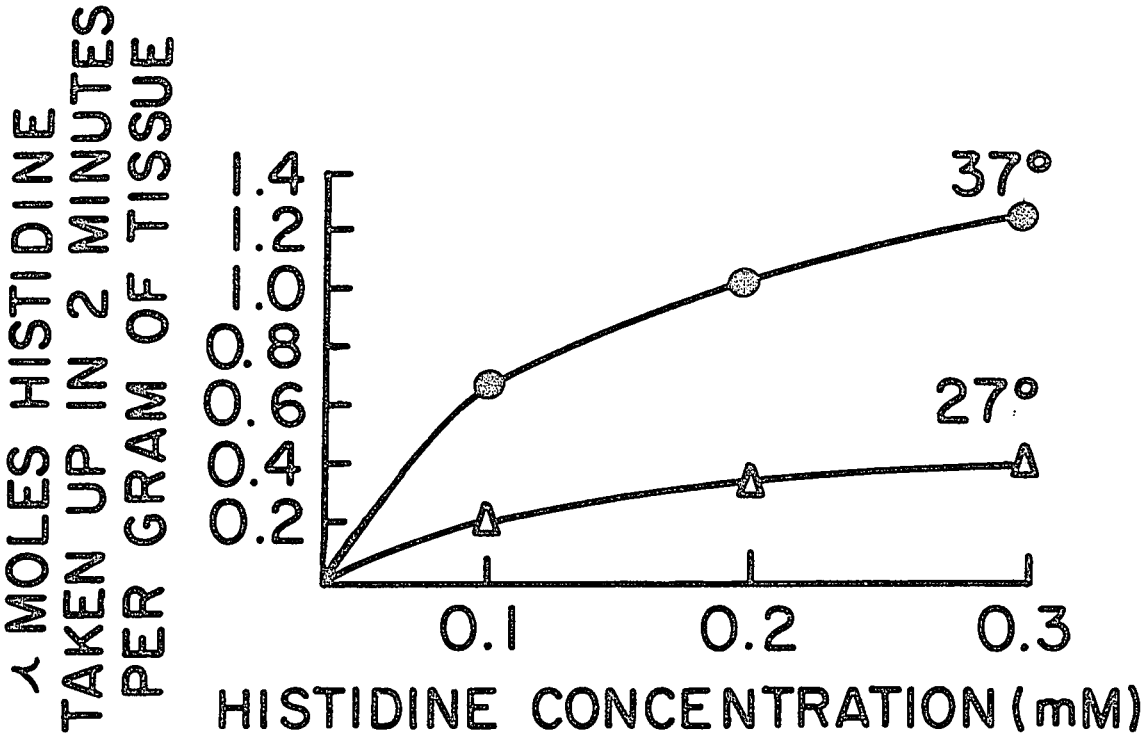
phenylalanine is in the neutral amino acid group, it is considered so here. Read, et al., (29) also found that histidine uptake, unlike that of any other amino acid, is inhibited by both arginine (basic amino acid) and phenylalanine (neutral amino acid) and that reciprocal inhibition by histidine also occurs. In order to clearly deliniate this apparently double entry route for histidine, the interactions of phenylalanine and arginine with histidine uptake have been examined in detail.

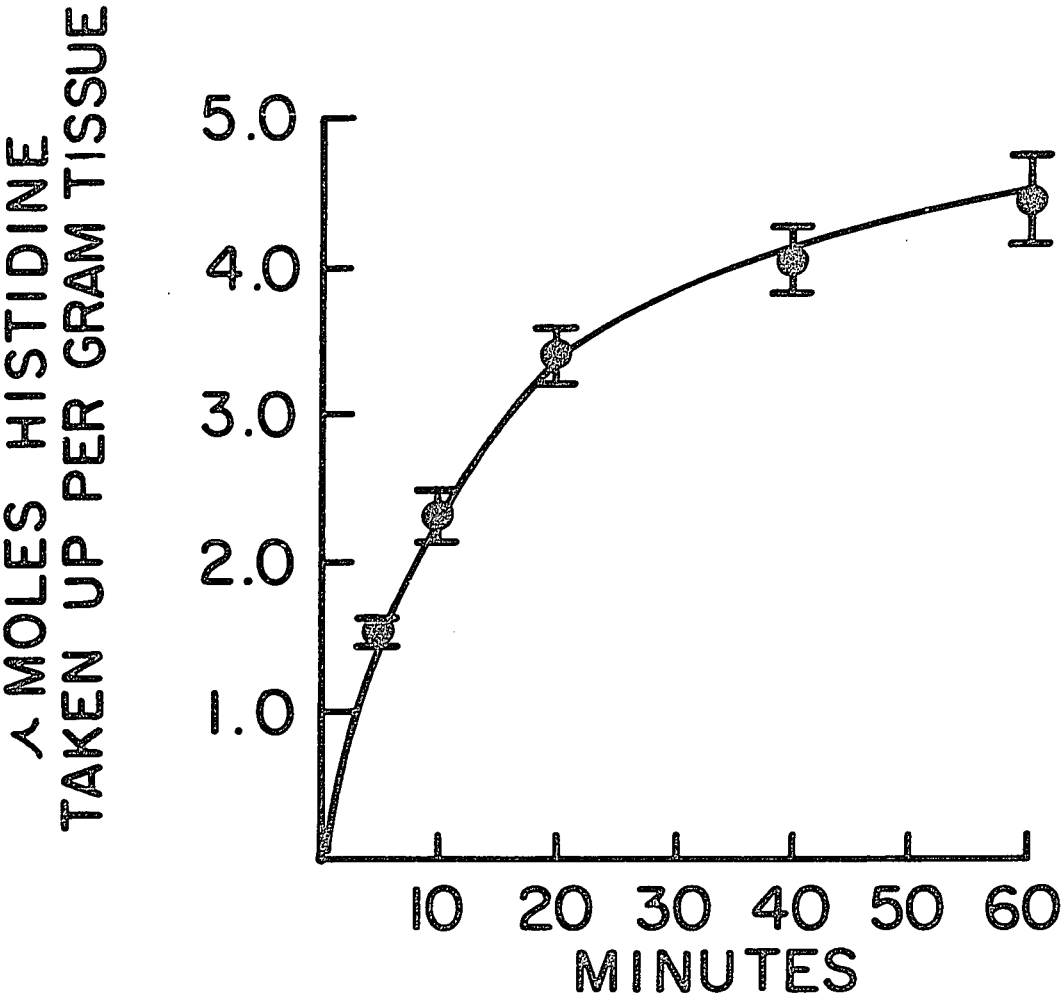
Hymenolepis accumulates histidine against a concentration difference reaching a minimal internal concentration of 1.1 mM histidine after 60 minutes incubation in 4 ml of 0.1 mM histidine initial concentration (i.e., 4.4 micromoles per gm dry weight = 4.4 micromoles per 4 gm worm water = 1.1 millimoles per 1000 gm worm water) (Fig. 1b). Assuming a homogeneous distribution of internal water and an average sample dry weight of 0.05 gm, the final concentration difference is 1.1 mM inside and 0.045 mM outside. If, inside the cells, there is compartmentalization of histidine the concentration difference is even greater.

An amino acid analysis of the ethanol extract of a one hour incubation shows about 20 ninhydrin positive peaks, but only the peak corresponding to histidine is radioactive. This shows that histidine is not degraded during the one hour incubation. Read, et al., (pers. comm.) have measured the incorporation of external radioactive histidine into Hymenolepis protein during a one hour incubation and found that incorporation is minimal. The accumulation is linear with time for approximately 20 minutes, after which

Fig. 1a. The uptake of histidine-^{14}C by Hymenolepis diminuta with increasing histidine-^{14}C concentration in the media. The incubations are for two minutes.

Fig. 1b. The uptake of histidine-^{14}C by Hymenolepis diminuta with increasing time. Initial histidine-^{14}C concentration 0.1 mM.


γ MOLES HISTIDINE TAKEN UP IN 2 MINUTES PER GRAM OF TISSUE
1.4
1.2
1.0
0.8
0.6
0.4
0.2
37°
27°
0.1
0.2
0.3
HISTIDINE CONCENTRATION (mM)


γ MOLES HISTIDINE TAKEN UP PER GRAM TISSUE
5.0
4.0
3.0
2.0
1.0
10
20
30
40
50
60
MINUTES

time it approaches a steady state (Fig. 1b). The initial rate of uptake is temperature dependent (Fig. 1a) and varies with the substrate concentration as a rectangular hyperbola, reaching a maximum velocity at about 2 mM histidine (Fig. 2, H curve).

Figure 3 shows the uptake kinetics of histidine in the presence of varying amounts of 1) arginine alone, 2) phenylalanine alone, and 3) both amino acids together. It is evident from the figure that about half the histidine uptake is inhibited by arginine and about half by phenylalanine. The presence of both arginine and phenylalanine are almost totally inhibitory, i.e., the curve plateaus about v_i = 0.15 micromoles histidine per 2 min per gm. The components which comprise this residual uptake are shown in the AP curve of Figure 2. The uptake kinetics for varying histidine concentrations in the presence of saturating amounts of both arginine and phenylalanine (AP) represent a measure of the "residual" component, showing a slight shoulder followed by a straight line. The linear portion of the curve is interpreted as uptake by diffusion, and the dotted line through the origin (same slope as the linear portion) is interpreted to be the diffusion component. The area between these two curves is probably due to some saturable system not inhibited by arginine or phenylalanine.

In Figure 2 the A curve represents the histidine transport-mediating system which is not inhibited by arginine and is, therefore, composed of the phenylalanine-sensitive histidine transport system plus the residual system (mostly diffusion) described above. If the AP curve (the residual system(s)) is subtracted from the A

Fig. 2. Effect of 4mM arginine (A), 4mM phenylalanine (P), or 4mM arginine plus 4mM phenylalanine (AP) on the uptake of histidine-^{14}C in two minute incubations. H = histidine uptake without inhibitor. See text for explanation of diffusion curve.

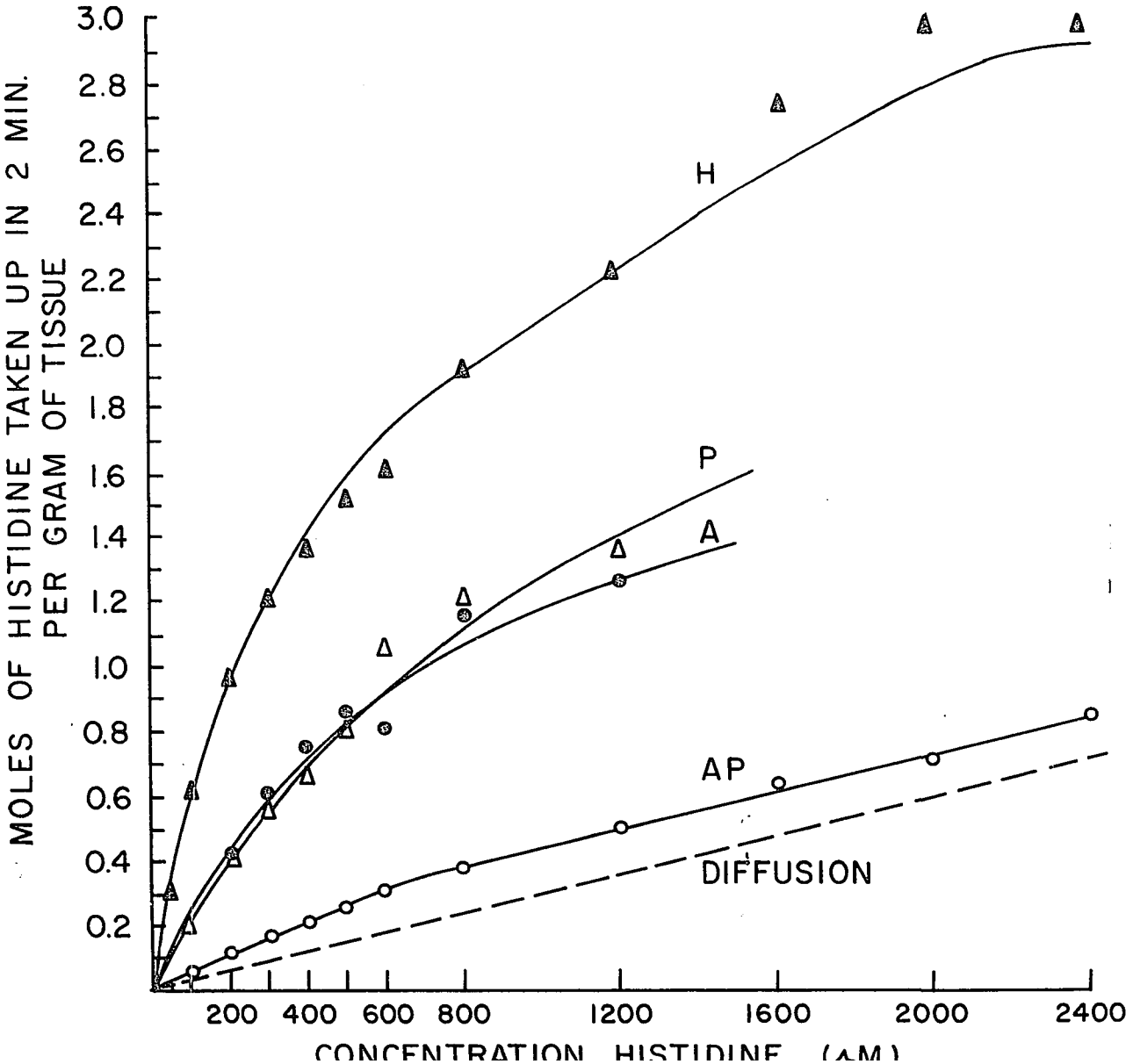
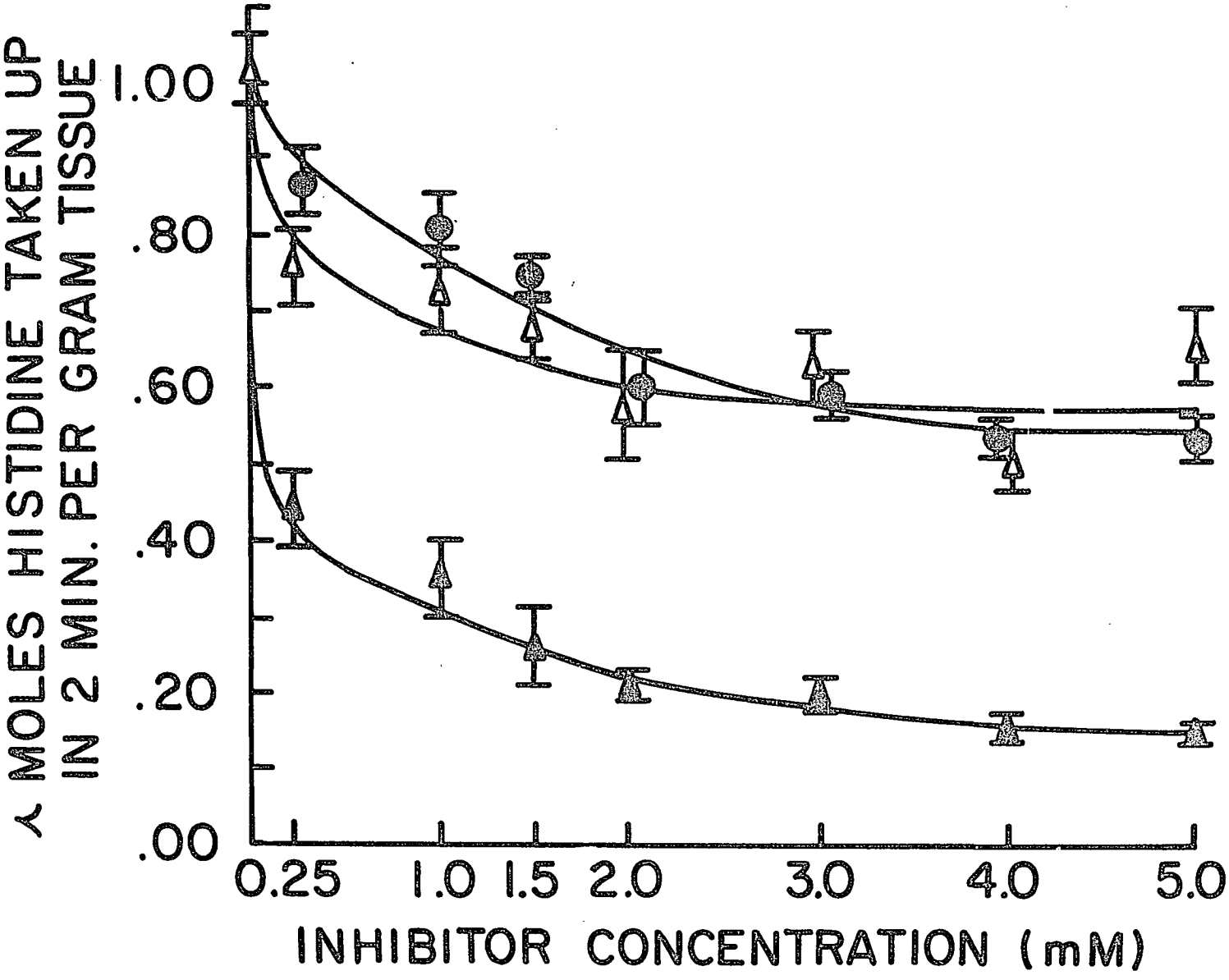

MOLES OF HISTIDINE TAKEN UP IN 2 MIN.
PER GRAM OF TISSUE
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
H
P
A
AP
DIFFUSION
200
400
600
800
1200
1600
2000
2400
CONCENTRATION HISTIDINE (μM)

Fig. 3. The inhibition of histidine-^{14}c uptake by increasing concentrations of phenylalanine (o), arginine (▲) , and phenylalanine plus arginine (▲). Histidine-^{14}C concentration was 0.3 mM. Incubations were for 2 minutes.


γ MOLES HISTIDINE TAKEN UP IN 2 MIN. PER GRAM TISSUE
1.00
.80
.60
.40
.20
.00
0.25
1.0
1.5
2.0
3.0
4.0
5.0
INHIBITOR CONCENTRATION (mM)

curve, then the kinetics for histidine entry through the phenylalanine-sensitive entry site can be isolated (Fig. 4, curve d). The same reasoning obtains for the P curve of Figure 2, i.e., if the AP curve is subtracted from the P curve, histidine entry through the arginine-sensitive system is isolated (Fig. 4, curve c). When the AP curve (Fig. 2) is subtracted from total histidine uptake (H curve, Fig. 2) curve a (Fig. 4) is obtained. If histidine entry is through two separate systems and if these have indeed been isolated kinetically, then the sum of curve c and d in Figure 4 should equal curve a. Curve b is the sum of curves c and d. The theoretical curve, b, is within 10 per cent of the actual curve, a, and considering that c and d both have standard deviations of 10 per cent, curves a and b are not considered to differ. These curves as well as those in Figures 2 and 5 were plotted by the IBM 7040 computer programmed for the best fit to a rectangular hyperbola by the method of least squares, giving the most weight to those points with the smaller standard deviation. Each point in Figure 2 represents the average of from 5 to 20 separate replications with most of the points being an average of 10 replications.

The histidine curves with and without inhibition give competitive type vertical intersection when plotted in a Lineweaver-Burk plot (Fig. 5). It is from these plots that the curves in Figure 2 were obtained. To test whether arginine and phenylalanine compete with histidine uptake in a fully competitive way, it is necessary to vary the concentration of one inhibitor keeping the concentration of histidine constant and the concentration of the other inhibitor

Fig. 4. Resolution of the arginine-sensitive and phenlyalanine-sensitive systems for histidine transport. A = H - AP of Fig. 2; B = Sum of C and D; C = P - AP of Fig. 2; D = A - AP of Fig. 2.

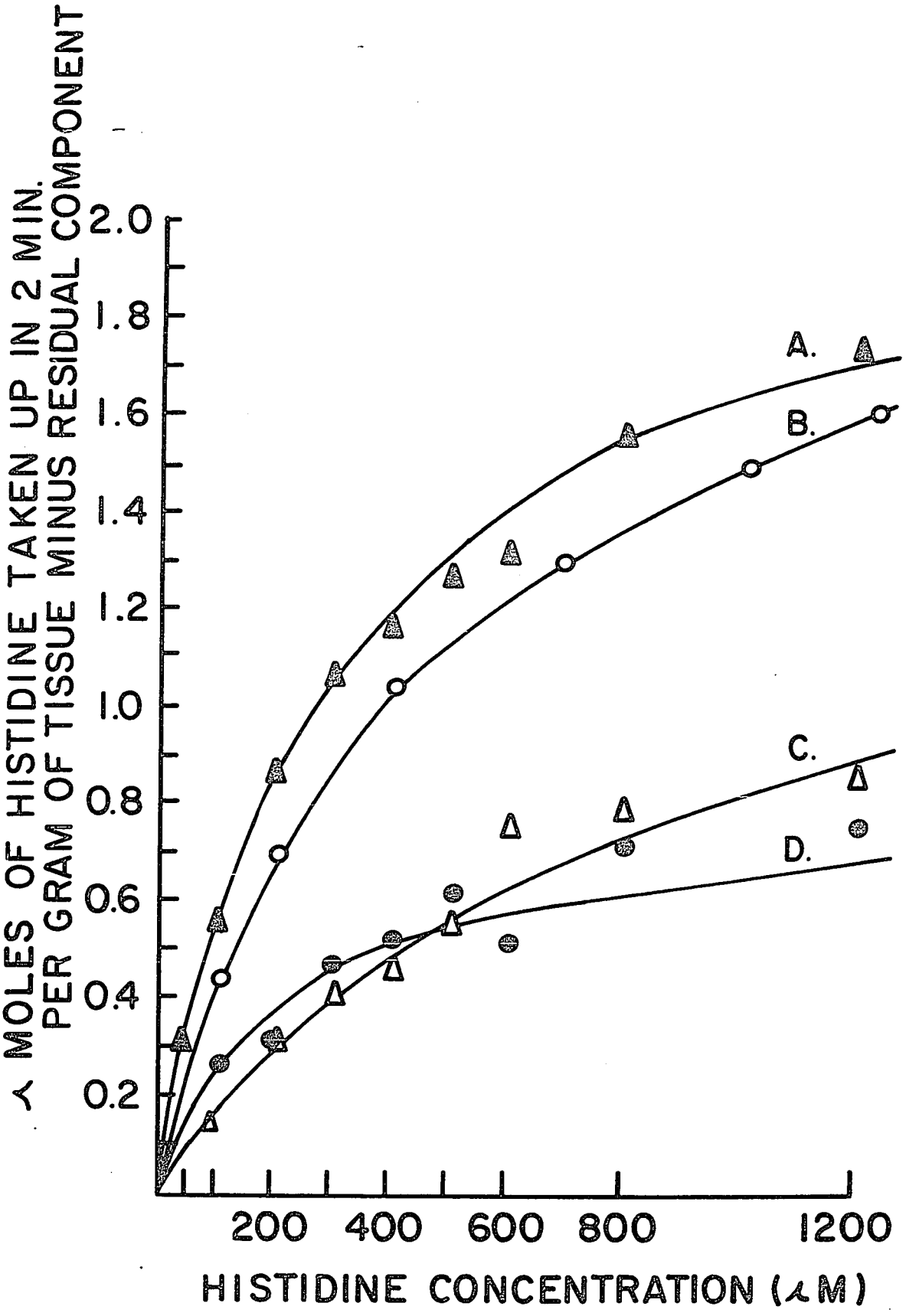
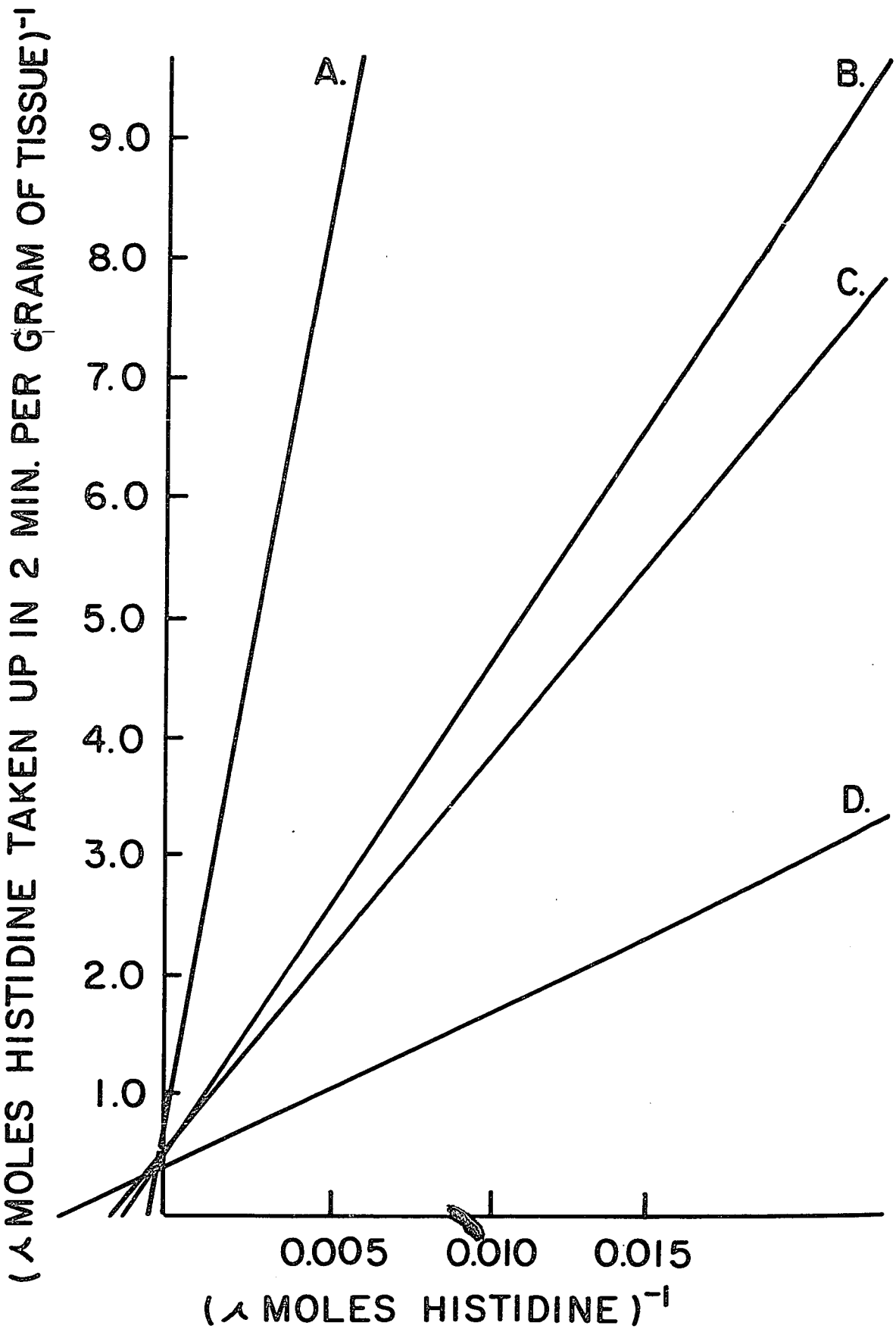

λ MOLES OF HISTIDINE TAKEN UP IN 2 MIN. PER GRAM OF TISSUE MINUS RESIDUAL COMPONENT
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
A.
B.
C.
D.
200
400
600
800
1200
HISTIDINE CONCENTRATION (μM)

Fig. 5. Lineweaver-Burk plots of histidine uptake in 2 minute incubations. A with 4 mM arginine plus 4 mM phenylalanine; B with 4 mM phenylalanine; C with 4 mM arginine; D without inhibitor. Each curve is plotted by the 7040 IBM computer from a least squares analysis of the data points presented in Fig. 2.


(λ MOLES HISTIDINE TAKEN UP IN 2 MIN. PER GRAM OF TISSUE)⁻¹
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
A.
B.
C.
D.
0.005
0.010
0.015
(λ MOLES HISTIDINE)⁻¹

constant and saturating. The top two curves in Figure 6 were obtained when this was done with 300 uM histidine. When the residual entry is subtracted (0.15 micromoles/ 2 min/gm) the two bottom curves were obtained. Justification for using the value, 0.15 micromoles/2 min/gm, as the measure of entry by the residual systems is as follows: 1) in Figure 3 the AP curve plateaus at 0.15 micromoles, 2) in Figure 2 the velocity, at 300 uM histidine, on the AP curve is 0.15 micromoles/2 min/gm, and 3) the plateaus of the two top curves of Figure 6 are at 0.15 micromoles/2 min/gm. If the two bottom curves of Figure 6 are replotted as 1/v vs inhibition concentration, two straight lines are obtained (Fig. 7). This indicates fully competitive inhibition (8).

That the inhibition in each isolated system is fully competitive is evidence that these two kinetically isolated systems correspond to neutral and basic amino acid transport-mediating systems. Further evidence for this conclusion is that alanine, a kinetically typical neutral amino acid, does not increase the inhibition observed with phenylalanine nor with alanine (Table II).

From the data presented above the following conclusions have been drawn: 1) histidine is taken up by Hymenolepis via neutral and basic amino acid transport-mediating systems, 2) the kinetics of histidine entry via the two systems have been isolated from one another, and 3) neutral and basic entry systems constitute the major routes of histidine entry into cells, but in addition to these routes and diffusion there is some entry through minor saturable system(s) which is not inhibited by the neutral or basic amino acids tested.

Fig. 6. Effect of increasing concentration of one inhibitor on histidine uptake with histidine and second inhibitor concentration constant. A = Varying phenylalanine concentration, 0.3 mM histidine, 4.0 mM arginine; B = varying arginine concentration; 0.3 mM histidine, 4.0 mM phenylalanine; C = same as curve A, corrected for residual uptake; D = same as curve B corrected for residual uptake. Incubation 2 minutes.

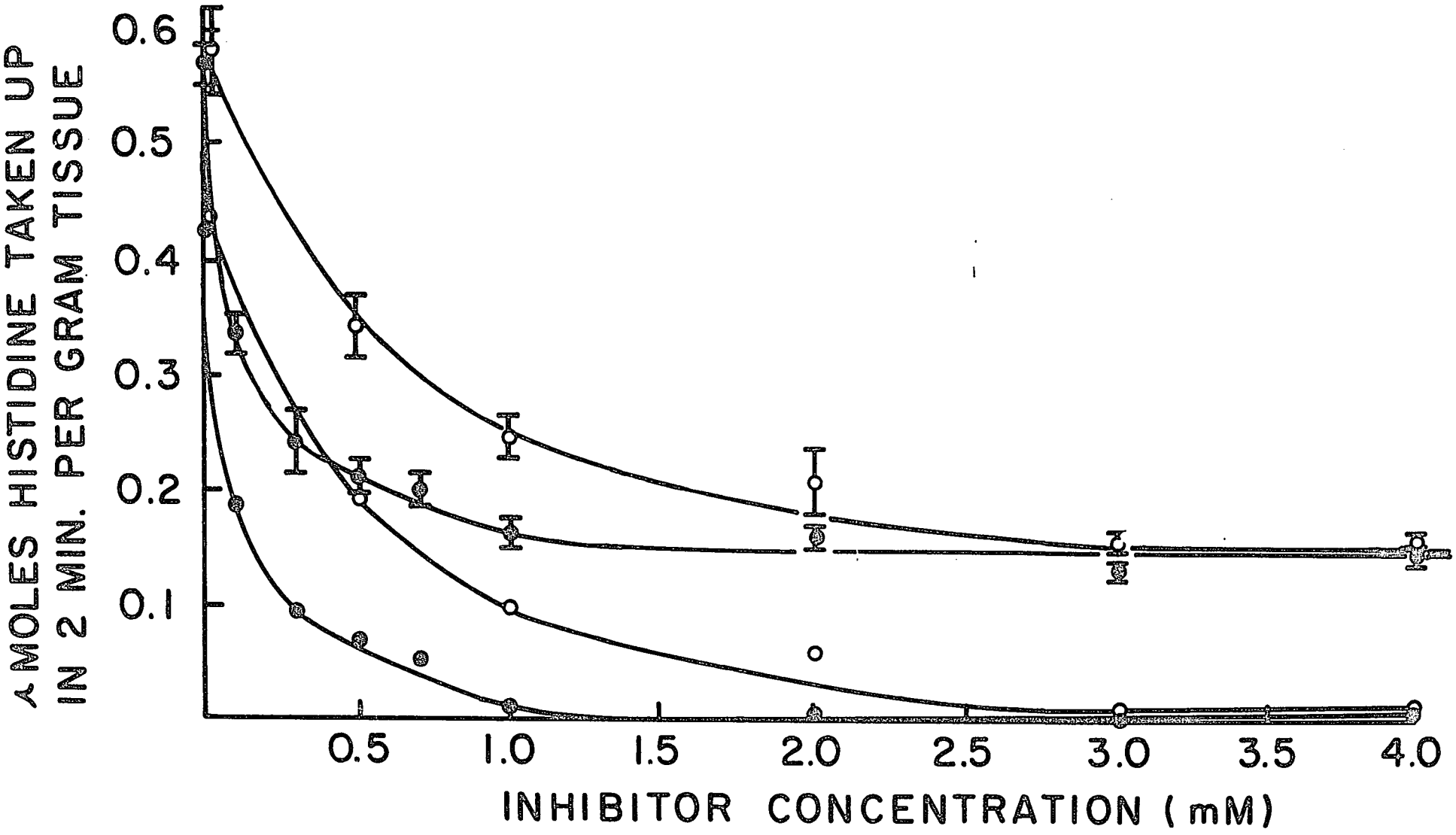

λ MOLES HISTIDINE TAKEN UP
IN 2 MIN. PER GRAM TISSUE
0.6
0.5
0.4
0.3
0.2
0.1
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
INHIBITOR CONCENTRATION (mM)

Fig. 7. Fully competitive inhibition in the isolated histidine transport systems.

(o) Isolated phenylalanine-sensitive histidine transport system. Media contained 0.3 mM histidine-^{14}C, 4.0 mM arginine and varying concentrations of phenylalanine.

(o) Isolated arginine-sensitive histidine transport system. Media contained 0.3 mM histidine-^{14}C, 4.0 mM phenylalanine, and varying concentrations of arginine. Incubation 2 minutes. These points are taken from the two bottom curves of Fig. 6.

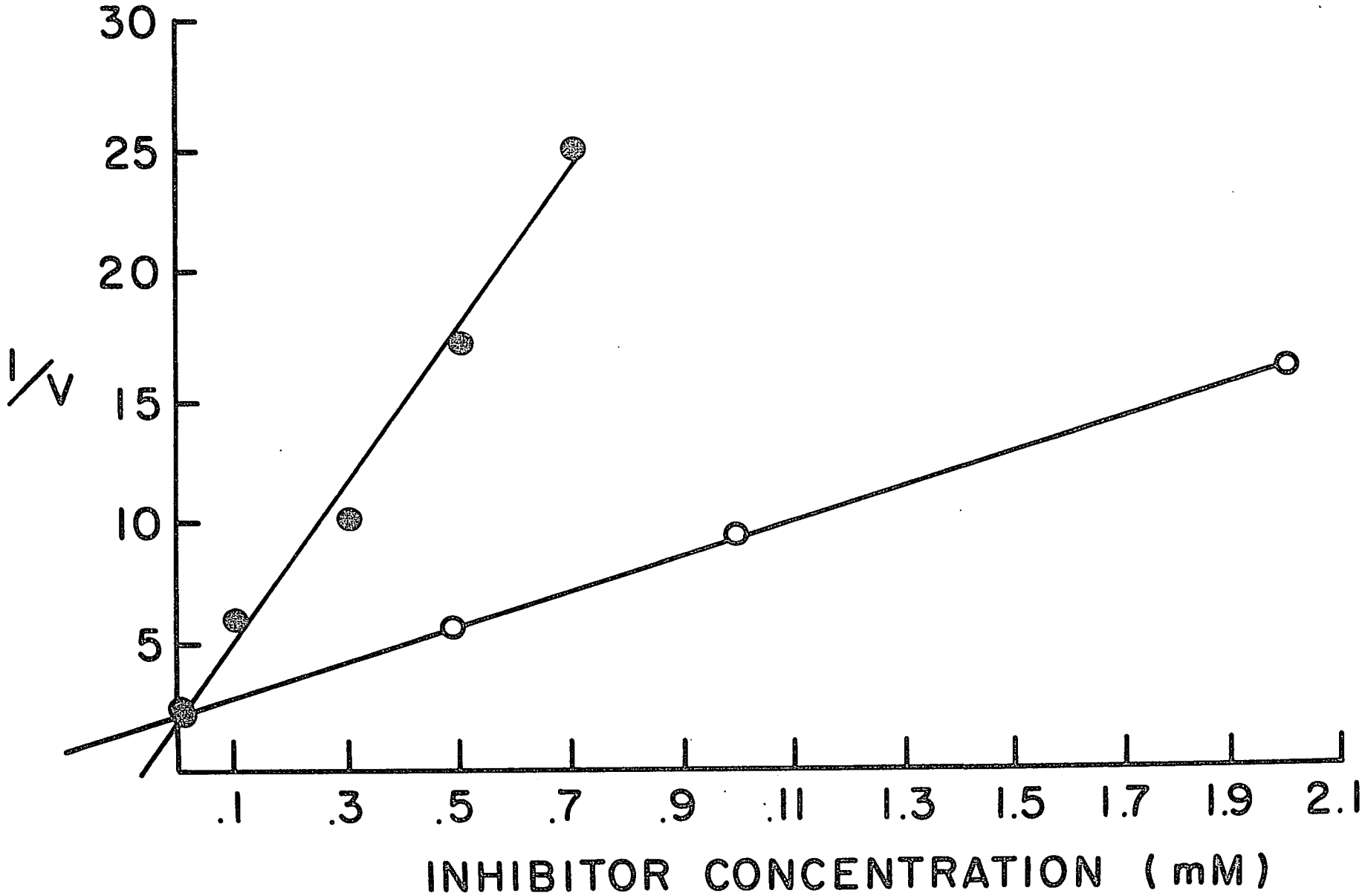

30
25
20
15
10
5
1/v
.1
.3
.5
.7
.9
.11
1.3
1.5
1.7
1.9
2.1
INHIBITOR CONCENTRATION (mM)

TABLE II

EFFECT OF ARGININE, PHENYLALANINE, AND ALANINE ON HISTIDINE TRANSPORT

The uptake of histidine-^{14}C initially at 0.3 mM was measured in 2 minute incubations. Uptake is expressed as micromoles histidine/gram dry weight/2 minutes.

Inhibitor in medium	Histidine uptake
None	1.21 ± 0.11
4 mM Arginine	0.54 ± 0.06
4 mM Phenylalanine	0.56 ± 0.05
4 mM Alanine	0.82 ± 0.09
4 mM Phenylalanine + 4 mM Alanine	0.60 ± 0.06
4 mM Phenylalanine + 4 mM Arginine	0.15 ± 0.009
4 mM Alanine + 4 mM Arginine	0.28 ± 0.03
4 mM Phenylalanine + 4 mM Alanine + 4 mM Arginine	0.13 ± 0.02

Inhibition by preloaded sugars on subsequent amino acid uptake in Hymenolepis has been reported (19, 30). In order to determine whether preloaded sugars have an effect on histidine uptake the experiments described in Table III were done. When both sugar and histidine were in the two minute incubation medium the sugar had no effect on histidine uptake. When the worms were preincubated with sugar and later incubated for two minutes with histidine there was an effect on histidine uptake and the effect varied with the time of preincubation. Preincubation in sugar for 10, 15 and 20 minutes (Table III) did not inhibit the subsequent uptake of histidine, but preincubation in sugar for 30 minutes inhibited histidine uptake about 20 per cent, compared to a sugarless preincubation medium.

Histidine uptake is affected to some extent by handling the worms, but since each preincubation treatment was accompanied by a KRT control the variation induced by handling does not affect comparisons.

Since the inhibition of histidine uptake, even at 30 minutes preincubation, was so slight studies using sugars as uptake inhibitors were discontinued.

ii) Efflux: The efflux of histidine can be studied by preloading Hymenolepis with histidine ^{14}C and measuring the disappearance of the radioactive histidine from the worms if the following three conditions are met: 1) histidine is not converted to other substances during the period of experimentation, 2) histidine inside the worms is not bound, e.g., incorporation into protein and 3) histidine inside the worms is located in a single compartment which is

TABLE III

EFFECT OF SUGARS ON THE UPTAKE OF HISTIDINE IN H. DIMINUTA

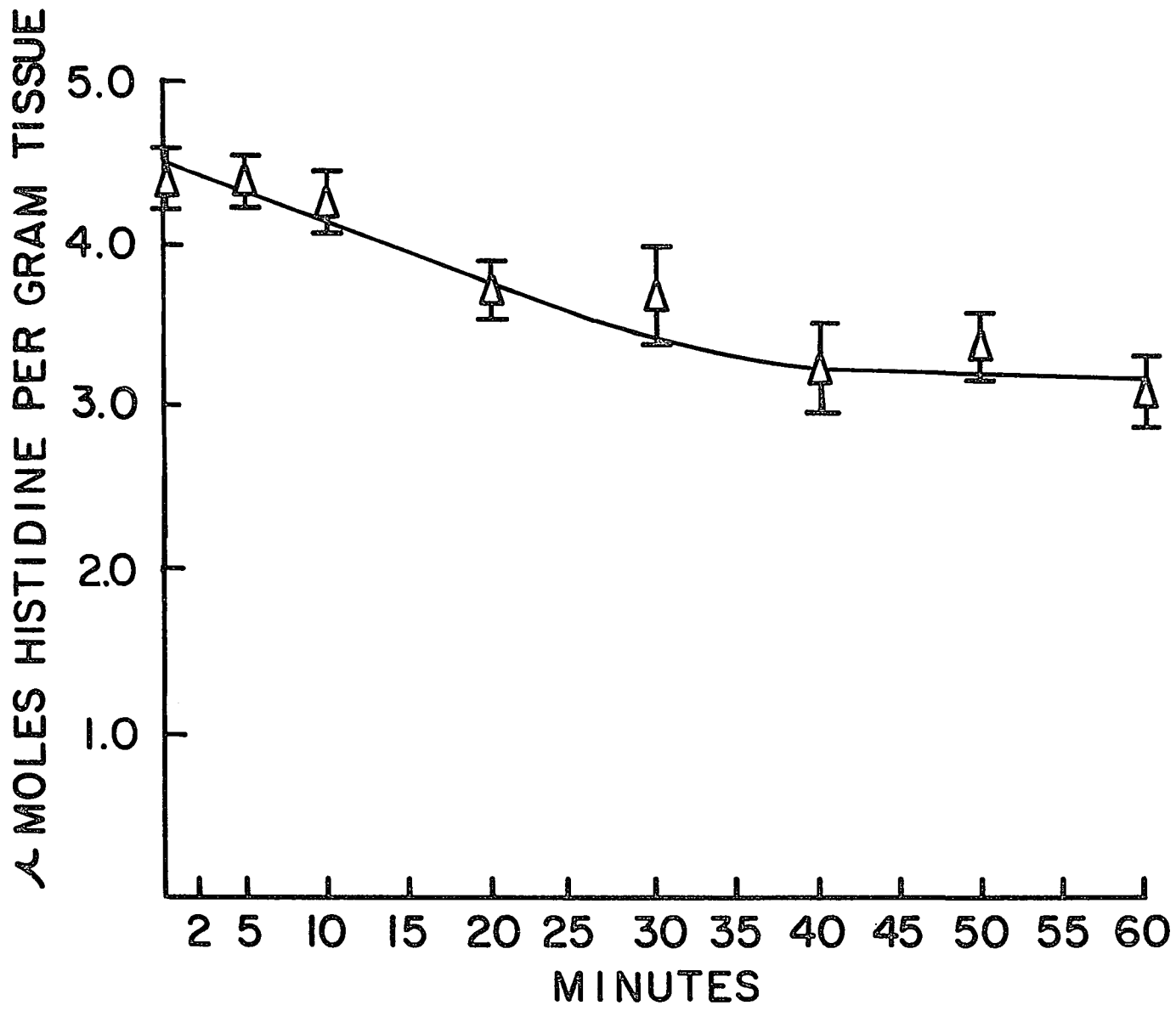
After the preincubation under the indicated conditions, samples were incubated for two minutes in the indicated incubation medium. Uptake is expressed as micromoles histidine-^{14}C/gram dry weight/2 minutes.

Preincubation Medium	Preincubation Time	Incubation Medium	Uptake
none	none	0.2 mM histidine	0.85 0.08
none	none	0.2 mM histidine 15 mM galactose	0.78 0.05
none	none	0.6 mM histidine	1.52 0.12
none	none	0.6 mM histidine 15 mM galactose	1.46 0.24
none	none	0.3 mM histidine	1.32 0.12
KRT	10 min	0.3 mM histidine	1.06 0.16
15 mM galactose	10 min	0.3 mM histidine	
KRT	15 min	0.3 mM histidine	1.34 0.12
15 mM galactose	15 min	0.3 mM histidine	1.08 0.09
KRT	20 min	0.3 mM histidine	1.15 0.12
15 mM galactose	20 min	0.3 mM histidine	1.07 0.06
KRT	30 min	0.3 mM histidine	1.32 0.07
15 mM galactose	30 min	0.3 mM histidine	1.03 0.14
15 mM glucose	30 min	0.3 mM histidine	1.08 0.08

in equilibrium with the inner surface of the membrane. Evidence that the first condition is met and the second partially fulfilled has been presented above. Concerning the third condition: If histidine enters more than one compartment during the incubation, then it can be assumed that these compartments might come to equilibrium with adjacent compartments at different rates. This might result in a biphasic curve for the efflux of preloaded ^{14}C from the worms. A biphasic curve has been observed in several cases by other investigators (with worms and other organisms) and has been interpreted to mean that compartmentalization occurs (19). During the periods of histidine efflux from Hymenolepis no such evidence for compartmentalization was observed (Fig. 8). Efflux shown in Figure 8 followed a 60 minute incubation in the presence of histidine ^{14}C. However, even in the absence of direct evidence for compartmentalization of histidine one cannot assume homogeneity of internal water in a complex organism like Hymenolepis. The most that can be assumed about the third condition is that a measure of accumulated histidine is directly and constantly proportional to the amount of histidine in the compartment (assumed to be homogeneous) which articulates directly with the outermost osmotic barrier. With these reservations, the efflux of histidine in Hymenolepis was studied.

The efflux of previously accumulated histidine in Hymenolepis is slow compared to its influx (compare Fig. 8 with Fig. 2). When the worms are preloaded in 100 uM histidine ^{14}C for 15 minutes followed by 15 minutes incubation in cold histidine, arginine or phenylalanine, efflux is stimulated as compared to efflux obtained

Fig. 8. Efflux of previously accumulated histidine-^{14}C in KRT. Samples preloaded by incubating 60 minutes in 0.1 mM histidine-^{14}C, then removing to efflux medium. Values are amounts of histidine-^{14}C remaining in the tissues.


μ MOLES HISTIDINE PER GRAM TISSUE
5.0
4.0
3.0
2.0
1.0
2 5 10 15 20 25 30 35 40 45 50 55 60
MINUTES

when the worms are incubated in KRT (Fig. 9). Table IV shows efflux in KRT alone.

As histidine in the medium is increased the efflux of pre-loaded histidine ^{14}C is stimulated until a plateau is reached (=700 uM histidine) (Fig. 9). Arginine or phenylalanine concentrations about 100 uM, however, do not further stimulate histidine efflux, i.e., both the arginine and the phenylalanine stimulating systems become saturated at concentrations below 100 uM. In Figure 9 the curve showing stimulation by arginine plus phenylalanine (AP curve) plateaus lower than either the curve of stimulation by arginine alone (A curve) or the one by phenylalanine alone (P curve). If the efflux in KRT (Table IV) represents diffusion and this value is subtracted from the A, P, and AP curves, the remaining A and P curves are additively equal to the remaining AP curve. From this it is concluded that the systems through which arginine and phenylalanine stimulate histidine efflux are kinetically independent and separate.

As can be seen in Figure 9, the stimulation of efflux of internal histidine by external histidine (H curve) plateaus lower than any of the other curves. Attempts were made to determine whether the stimulation of efflux of histidine by exogenous histidine occurs via the same or different systems than those of arginine and phenylalanine. The experiment was as follows: efflux of histidine ^{14}C was measured in the presence of increasing histidine concentrations 1) with histidine alone, 2) with 1250 uM arginine added to the histidine, 3) with 1250 uM phenylalanine added, and 4) with 1250 uM of both arginine and phenylalanine added (Fig. 10). The

Fig. 9. Effect of external medium concentration of phenylalanine, arginine, phenylalanine plus arginine, and histidine on the efflux of previously accumulated histidine-^{14}C. Samples preloaded by incubating 15 minutes in 0.1 mM histidine-^{14}C, then removing to efflux media for 15 minutes. A = arginine, P = phenlyalanine, AP = arginine plus phenylalanine (each at indicated concentration), H = histidine. Values are amounts of histidine-^{14}C remaining in the tissues after 15 minute efflux.

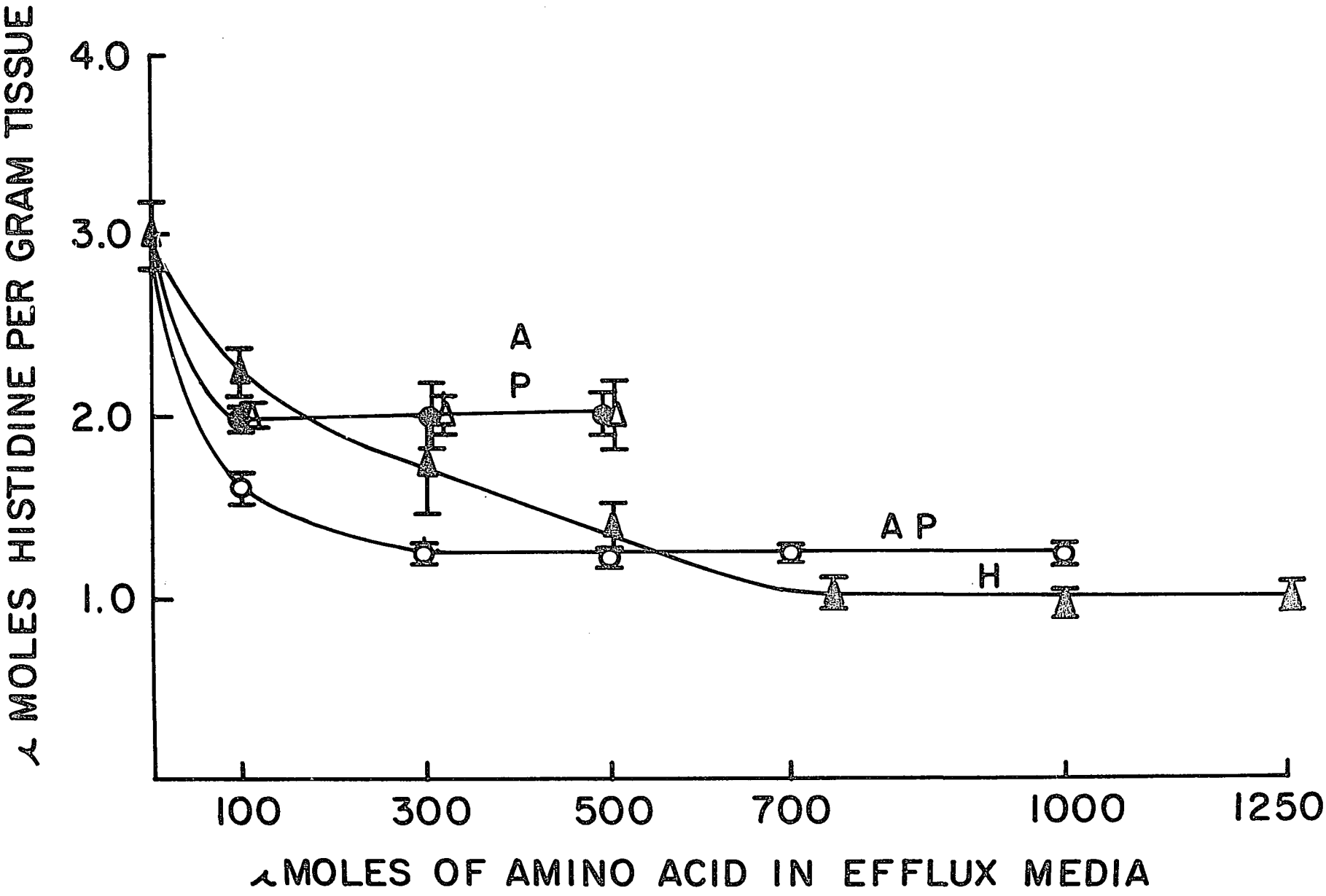

μ MOLES HISTIDINE PER GRAM TISSUE
4.0
3.0
2.0
1.0
A
P
AP
H
100
300
500
700
1000
1250
μ MOLES OF AMINO ACID IN EFFLUX MEDIA

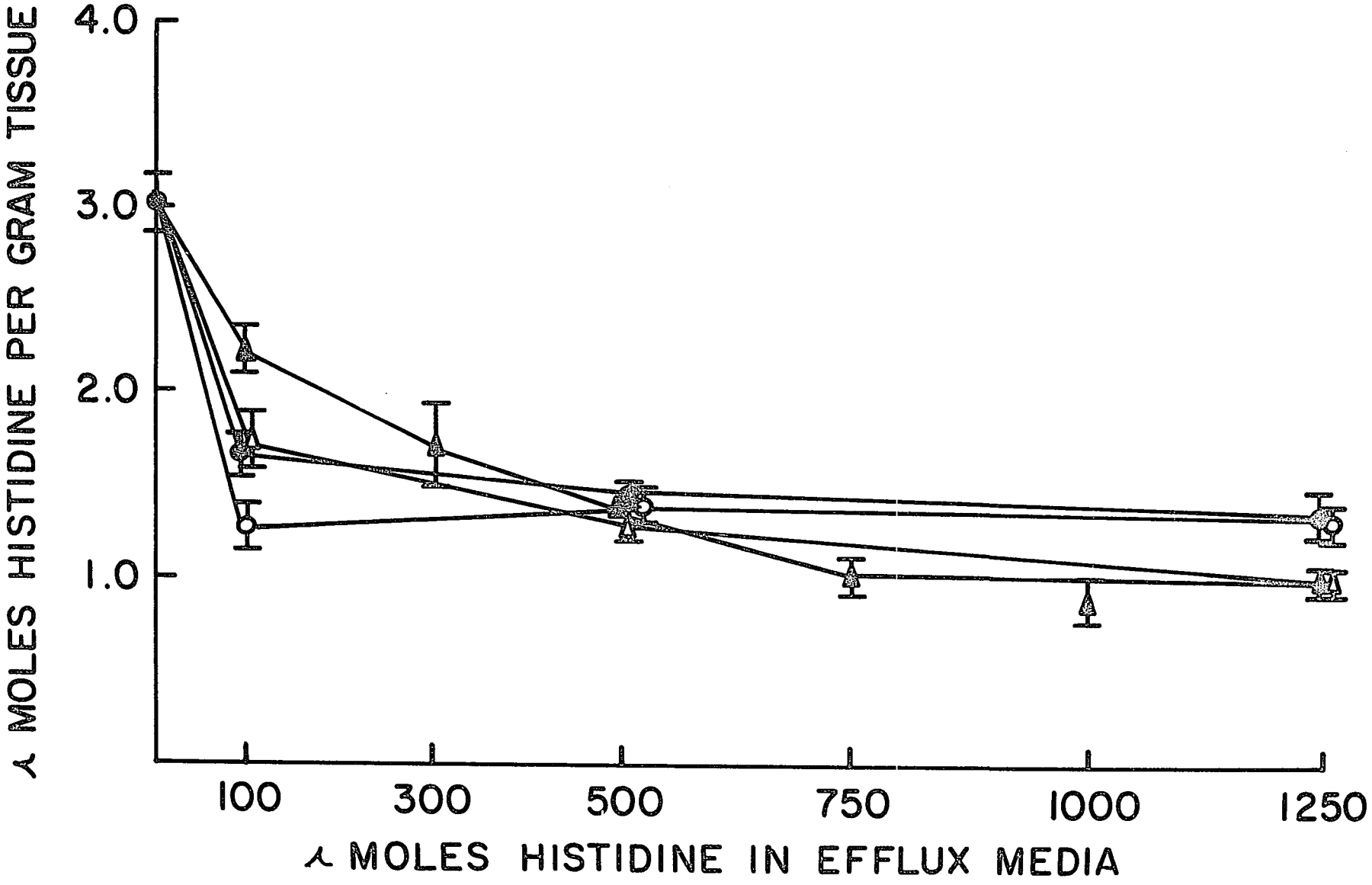
TABLE IV

EFFECT OF TEMPERATURE ON THE EFFLUX OF PRELOADED HISTIDINE

Samples were preincubated at 37° for 15 minutes in 0.1 mM histidine-^{14}C, rinced in KRT, and incubated in KRT for 15 minutes at the temperatures indicated. Residual histidine-^{14}C is the amount remaining in the tissues after incubation expressed as micromoles/gram dry weight.

Temperature of Incubation Medium	Residual Histidine-14C
no incubation	3.0 ± 0.2
37°	2.5 ± 0.3
25°	2.7 ± 0.3

Fig. 10. Effect on efflux of previously accumulated histidine-^{14}C of external medium concentration of histidine (▲), histidine + 1.25 mM phemlyalanine (△) , histidine + 1.25 mM arginine (●), histidine + 1.25 mM phenlyalanine + 1.25 mM arginine (○). The values for these points represent three experiments with 15 replications per experiment. Samples were preloaded by incubating 15 minutes in 0.1 mM histidine-^{14}C, then removed to efflux media ofr 15 minutes.


μ MOLES HISTIDINE PER GRAM TISSUE
4.0
3.0
2.0
1.0
100
300
500
750
1000
1250
μ MOLES HISTIDINE IN EFFLUX MEDIA

data presented in Figure 10 are interpreted to mean that histidine stimulates efflux through the same systems affected by arginine and phenylalanine, and only through these systems. The reasons for this conclusion are the following: 1) at exogenous concentrations of 100 uM histidine the addition of arginine or phenylalanine enhances efflux (Fig. 10) more than when histidine is absent (Fig. 9), and the addition of both simultaneously increases stimulation only to the plateau achieved in the AP curve (Fig. 9). If histidine stimulation of efflux were occurring as a result of a third system, the addition of arginine and phenylalanine in the presence of histidine would show a greater stimulation than arginine and phenylalanine alone, 2) at histidine concentrations of 500 uM the addition of arginine and/or phenylalanine has no effect (Fig. 10), i.e., at this concentration of histidine both the arginine system and the phenylalanine system are saturated. Again, if the histidine system were different one would expect an additive effect, 3) when the exogenous concentration of histidine is 1250 uM, the addition of arginine or arginine + phenylalanine, but not phenylalanine alone, decreases stimulation. (Fig. 10. The values for these points represent three experiments with 15 replications per experiment.) These data indicate that although arginine and phenylalanine possess greater affinities for their respective systems than histidine, arginine is a less effective stimulator of histidine efflux than either phenylalanine or histidine.

To further test these conclusions the experiments of Figure 10 were repeated with higher and lower internal concentrations of

histidine. At the lower internal concentrations of histidine, obtained by preincubating in 100 uM histidine for 5 rather than 15 minutes (Table V), the pattern was the same as observed in the data of Figure 10. At higher internal concentrations, obtained by preincubating in 300 uM histidine for 15 minutes (Table VI), there is no effect on stimulation by exogenous histidine when arginine or phenylalanine is added. At a given concentration of external histidine the values overlap whether or not arginine and/or phenylalanine is added. Increasing the concentrations of arginine and phenylalanine to 2.6 mM did not increase stimulation above the value obtained with histidine alone. In Table VI it also can be seen that increases of external histidine beyond 250 uM do not increase the efflux of histidine; it takes less histidine to saturate the exogenous stimulating system when the internal concentration of histidine is high than when the internal concentration is low. Thus, the stimulation of efflux of internal histidine is dependent not only upon the quality and quantity of exogenous amino acids but also on the internal concentration of histidine. Also it is evident that when the arginine and phenylalanine systems are saturated with histidine, arginine and phenylalanine are ineffective in changing efflux, but both amino acids have a decided effect on histidine efflux when administered alone (Table VII). Table VII verifies the fact that the concentrations of arginine and phenylalanine used in the experiments described in Table VI are saturating.

Other cases of stimulation of transmembrane movements by amino acids on the opposite side of the membrane have been reported

TABLE V

EFFECT OF HISTIDINE, ARGININE, AND PHENYLALANINE ON EFFLUX OF HISTIDINE-^{14}C AFTER FIVE MINUTE PRELOADING

Samples were preincubated for 5 minutes in 0.1 mM histidine-^{14}C, rinsed in KRT, and incubated for 15 minutes in media indicated. Residual histidine-^{14}C is the amount remaining in the tissues after incubation, expressed as micromoles/gram dry weight.

Incubation Medium	Residual Histidine-^{14}C
No incubation	2.00 ± 0.24
0.10 mM Histidine	1.57 ± 0.23
0.10 mM Histidine + 1.25 mM Arginine	1.19 ± 0.07
0.10 mM Histidine + 1.25 mM Phenylalanine	1.19 ± 0.14
0.10 mM Histidine + 1.25 mM Arginine + 1.25 mM Phenylalanine	0.90 ± 0.07
0.50 mM Histidine	1.01 ± 0.10
1.25 mM Histidine	0.84 ± 0.07
1.25 mM Histidine + 1.25 mM Arginine	0.97 ± 0.05
1.25 mM Histidine + 1.25 mM Phenylalanine	0.75 ± 0.10
1.25 mM Histidine + 1.25 mM Arginine + 1.25 mM Phenylalanine	0.95 ± 0.08

TABLE VI

EFFECT OF HISTIDINE, ARGININE, AND PHENYLALANINE ON EFFLUX OF HISTIDINE-^{14}C AFTER FIFTEEN MINUTE PRELOADING

Samples were preincubated for 15 minutes in 0.3 mM histidine-^{14}C, rinsed in KRT, and incubated for 15 minutes in media indicated. Residual histidine-^{14}C is the amount remaining in the tissues after incubation, expressed as micromoles/gram dry weight.

Incubation Medium	Residual Histidine-^{14}C
No incubation	5.40 ± 0.51
KRT	4.36 ± 0.42
0.25 mM Histidine	3.19 ± 0.53
0.25 mM Histidine + 1.25 mM Arginine	2.68 ± 0.12
0.25 mM Histidine + 1.25 mM Phenylalanine	2.60 ± 0.06
0.50 mM Histidine	2.74 ± 0.23
0.50 mM Histidine + 1.25 mM Arginine	2.57 ± 0.14
0.50 mM Histidine + 1.25 mM Phenylalanine	2.50 ± 0.23
0.50 mM Histidine + 1.25 mM Arginine + 1.25 mM Phenylalanine	2.47 ± 0.16
1.00 mM Histidine	2.05 ± 0.30
1.00 mM Histidine + 1.25 mM Arginine	2.45 ± 0.17
1.00 mM Histidine + 1.25 mM Phenylalanine	1.85 ± 0.26
1.00 mM Histidine + 1.25 mM Arginine + 1.25 mM Phenylalanine	2.32 ± 0.02
1.25 mM Histidine	2.13 ± 0.26
1.25 mM Histidine + 1.25 mM Arginine + 1.25 mM Phenylalanine	2.34 ± 0.10
1.25 mM Histidine + 2.60 mM Arginine + 2.60 mM Arginine	2.38 ± 0.24

TABLE VII

EFFECT OF ARGININE OR PHENYLALANINE ON EFFLUX OF HISTIDINE-^{14}C

Conditions of the experiment as in Table VI

Incubation Medium	Residual Histidine-^{14}C
No Incubation	6.22 ± 0.33
KRT	5.10 ± 0.57
0.5 mM Arginine	3.73 ± 0.17
1.25 mM Arginine	3.97 ± 0.37
0.5 mM Phenylalanine	4.03 ± 0.19
1.25 mM Phenylalanine	3.84 ± 0.16

in the literature. Heinz and Walsh (15) described what they called exchange diffusion wherein the uptake of amino acids from the external medium is stimulated by high concentrations of amino acids of the same mutually inhibitory transport group inside the cells. They also found that efflux is stimulated by exogenous amino acids of the same transport family. Their explanation of exchange diffusion is that the exit of an internal amino acid makes available a carrier for the entry of an external amino acid. The newly exited amino acid may return into the cell on the same carrier, but on reaching the outside of the membrane it becomes highly diluted. But, the main point here is that the stimulation is only apparent, not real, since there is no net gain of endogenous amino acids.

Working with methionine in Hymenolepis and valine in Calliobothrium, Read, et al., (29) found that efflux is markedly stimulated by external amino acids of the same transport family. They also found that preloading with any amino acid did not effect the subsequent uptake of the amino acid. They called this a modified form of exchange diffusion without presenting a new model to explain the assymmetry.

Kilejian (19) found, in Hymenolepis, that efflux of preloaded proline is stimulated by exogenous methionine, an amino acid known to compete for proline entry. She was of the opinion that her results could be explained as exchange diffusion; however, she found that preloading the cells with alanine, glycine, phenylalanine or proline, all inhibitors of proline uptake, did not stimulate uptake of proline, but that preloading the cells with hydroxyproline, another

inhibitor of proline entry, did stimulate subsequent uptake of proline. She did not test the effect of internal methionine on proline uptake.

Jacques (18), working with Ehrlich cells, reported that internal amino acids stimulate entry of L-tryptophane, and that these same amino acids stimulate entry when incubated simultaneously. After his kinetic analyses of the data he concluded that stimulation by preloaded amino acids is explained by a 1:1 amino acid:carrier ratio, whereas stimulation by simultaneously incubated amino acids demands a 2:1 amino acid:carrier ratio.

Paine and Heinz (25), working with Ehrlich cells, and Oxender and Christensen (24) reported that cells preloaded with amino acids show a reduction of subsequent uptake of amino acids of the same inhibitory group. Paine and Heinz explain their data by stating that the exit of amino acids was sufficiently rapid to provide competition with external amino acids for carrier.

Studying sugar uptake in red blood cells, Rosenberg (32) observed what he calls asymmetrical inhibition, i.e., the inhibition of efflux of previously accumulated glucose by external polyphloretine phosphate. Inhibition is asymmetrical because external polyphloretine does not inhibit the uptake of glucose. To explain this phenomenon he proposes an equilibrium between two forms of the carrier-inhibitor complex such that on one side of the membrane the affinity of the complex for the substrate is different than the affinity of the carrier alone, while on the other side of the membrane their affinities for the substrate are the same.

Exchange diffusion as described by Heinz and Walsh cannot explain the data reported here, for to do so one would expect preloading of cells with histidine, arginine or phenylalanine to enhance subsequent histidine uptake, and it is clear that this does not happen (Table VIII). It is also apparent that the phenomenon is not due to the action of exogenous amino acids on the inner surface of the membrane barrier since 1) preincubation for 2 minutes shows the same effect as preincubation for 15 minutes (Table IX). 2) Preloading with histidine + arginine or phenylalanine does not increase the efflux obtained with KRT alone (Table X). Efflux is increased only if histidine, arginine or phenylalanine is present on the outside of the membrane.

The following conclusions were drawn from the above data measuring efflux: 1) The efflux of internal histidine is stimulated by arginine, phenylalanine and histidine, and the mechanism involves the action of these amino acids on the outer surface of the membrane barrier, 2) the stimulation by arginine and phenylalanine occurs at two kinetically separate sites, and the stimulation by histidine occurs at both of these sites, 3) the affinity of arginine and phenylalanine for their respective sites is greater than the affinity of histidine for either site, yet the stimulation effect at the arginine site is greater if histidine is bound than if arginine is bound. This difference is not seen at the phenylalanine site.

It is tempting to speculate that the stimulation sites are the same as the entry sites. Two observations support this conclusion: 1) histidine acts at two kinetically differentiable sites in both entry and stimulation of efflux, and in both cases one site

TABLE VIII

EFFECT OF PRELOADING WITH HISTIDINE, ARGININE, OR PHENYLALANINE ON UPTAKE OF HISTIDINE-^{14}C

Samples were preincubated in the indicated media for 15 minutes, rinsed in KRT for 1 minute, and incubated in the indicated concentrations of histidine-^{14}C for 2 minutes. Uptake is expressed as micromoles histidine-^{14}C/gram dry weight/2 minutes.

Preincubation Medium	Incubation Medium	Histidine Uptake
KRT	0.10 mM Histidine-^{14}C	0.67 ± 0.04
0.1 mM Histidine	"	0.64 ± 0.03
0.1 mM Arginine	"	0.61 ± 0.07
0.1 mM Phenylalanine	"	0.67 ± 0.06
0.1 mM Arginine + 0.1 mM Phenylalanine	"	0.61 ± 0.03
KRT	0.45 mM Histidine-^{14}C	1.54 ± 0.19
0.1 mM Histidine	"	1.70 ± 0.11
0.1 mM Arginine	"	1.59 ± 0.13
0.1 mM Phenylalanine	"	1.59 ± 0.14
0.1 mM Arginine + 0.1 mM Phenylalanine	"	1.52 ± 0.12

TABLE IX

EFFECT OF HISTIDINE, ARGININE AND PHENYLALANINE ON PRELOADED HISTIDINE-^{14}C EFFLUX IN TWO MINUTES

Samples were preincubated in 0.1 mM histidine-^{14}C, rinsed in KRT for 1 minute, and incubated in the indicated concentrations of histidine, arginine and phenylalanine for 2 minutes. Residual histidine-^{14}C is the amount remaining in the tissues after incubation, expressed as micromoles/gram dry weight.

Incubation Medium	Residual Histidine-^{14}C
No incubation	2.99 ± 0.31
KRT	3.06 ± 0.35
0.5 mM Histidine	2.83 ± 0.24
0.75 mM Histidine	3.00 ± 0.25
1.0 mM Histidine	2.68 ± 0.18
1.25 mM Histidine	1.80 ± 0.08
1.25 mM Histidine + 0.5 mM Arginine + 0.5 mM Phenylalanine	2.10 ± 0.15

TABLE XI

EFFLUX OF HISTIDINE-^{14}C PRELOADED SIMULTANEOUSLY WITH ARGININE OR PHENYLALANINE

Samples were preincubated in the indicates media for 15 minutes, rinsed in KRT for 1 minute, and incubated in the indicated media for 15 minutes. Residual histidine-^{14}C is the amount remaining in the tissues after incubation, expressed as micromoles/gram dry weight.

Preincubation Medium	Incubation Medium	Residual Histidine-^{14}C
0.3 mM Histidine-^{14}C + 0.5 mM Arginine	No incubation	2.46 ± 0.30
"	KRT	2.02 ± 0.38
"	0.5 mM Histidine	1.41 ± 0.27
"	0.5 mM Arginine	1.88 ± 0.37
"	0.5 mM Phenylalanine	1.44 ± 0.16
0.3 mM Histidine-^{14}C + 0.5 mM Phenylalanine	No incubation	3.21 ± 0.39
"	KRT	2.94 ± 0.20
"	0.5 mM Histidine	1.78 ± 0.21
"	0.5 mM Arginine	2.25 ± 0.21
"	0.5 mM Phenyl-alanine	2.61 ± 0.16

shows affinity for arginine and one for phenylalanine, 2) the affinities of these sites are greater for arginine and phenylalanine than for histidine in both entry and efflux stimulation. Table I shows that the arginine entry site is saturated before 500 uM arginine, and Read (29) has shown that the phenylalanine entry site is saturated at ~600 uM phenylalanine. The isolated arginine-inhibited histidine entry site has a K_m of 750 uM histidine, and the phenylalanine-inhibited histidine entry site has a K_m of 1343 uM histidine (Fig. 4).

B. Neurospora crassa

i) A valid measure of amino acid uptake is growth of a nutritional mutant, deficient in its ability to synthesize the amino acid, in medium supplemented with the amino acid. That this is true has been demonstrated by Mathieson and Catcheside (22) and by DeBusk and coworkers (12).

Mathieson and Catcheside (22) demonstrated that the growth of certain histidine-requiring mutants of Neurospora is partially reduced when one of the following amino acids is present in the histidine supplemented growth media: Arginine, methionine, lysine, tryptophane, glycine, phenylalanine and leucine, and completely inhibited when one of the following pairs of amino acids is present in the histidine supplemented growth medium: Arginine + methionine, arginine + tryptophane, arginine + glycine, lysine + methionine, lysine + tryptophane or lysine + glycine. They also showed: 1) The degree of inhibition of growth of a given histidine mutant by these amino acids is a function of the genetic background of the mutant.

2) The effect of the inhibition is to prevent the uptake of histidine from the growth medium rather than to interfere with its utilization. Histidine is accumulated by the mutants against a concentration difference, and as growth proceeds the histidine gradually disappears from the mycelia. The addition of inhibitors following the accumulation of histidine has no effect upon its subsequent utilization, as judged by growth. 3) Wild type mycelia, as well as the histidine-requiring mutants, accumulate histidine from the growth medium, and the addition of arginine and methionine inhibits histidine accumulation. From this they concluded that the mutation causing the histidine requirement did not create the inhibition phenomenon but made it more easy to observe.

Dicker and DeBusk (12) studying the uptake of phenylalanine by conidia of wild type Neurospora found that the amino acids inhibiting uptake fall into the same groups as those inhibiting growth of phenylalanine-requiring mutants (3). They also conclude that in amino acid-requiring mutants the inhibiting amino acids interfere with uptake rather than with the utilization of the required amino acid.

In Neurospora, as in all other tissues studied, the neutral and the basic amino acids comprise two separate transport families based on mutual inhibition of uptake. DeBusk and coworkers have studied the uptake of phenylalanine and of arginine in Neurospora and have found that neutral but not basic amino acids are good inhibitors of phenylalanine uptake, and that the opposite is true for the inhibition of arginine uptake. It has repeatedly been found in

Neurospora that the growth of lysine-requiring mutants is inhibited by arginine (14) and that the growth of argining-requiring mutants is inhibited by lysine (34), but neither of these types of mutants is inhibited by neutral amino acids. Using canavanine resistant mutants, Bauerle and Garner (2) showed that arginine inhibits lysine uptake and vice versa. The mtr mutants of Stadler (35) have lost the ability to take up phenylalanine, and many other neutral amino acids, but arginine and lysine uptake was unimpaired.

Since it has been demonstrated 1) that growth of an amino acid-requiring mutant is a measure of uptake of that amino acid, and 2) that the neutral and the basic amino acids comprise two separate transport families in Neurospora, the data presented in Table XI and by Mathieson and Catcheside permit the following conclusions: 1) histidine enters Neurospora mycelia via the basic amino acid entry site, represented by arginine, and the neutral amino acid entry site, represented here by methionine, and 2) the two entry sites are separate as they are in Hymenolepis.

Table XI illustrates growth inhibition of 4 histidine-requiring mutants by several amino acids. The H3 mutants are missing histidine dehydrogenase, some earlier enzyme, or both (37). It is not known where the H6 mutants are blocked. The H3 mutants, K26 and K34, were subjected to more intensive study since these mutants were used in the genetic analyses described below. It can be seen in Table XI that growth of histidine-requiring mutants, in the presence of histidine, is inhibited when a neutral and a basic amino acid are added to the growth medium, but that growth is observed

TABLE XI

GROWTH OF HISTIDINE-REQUIRING MUTANTS IN MINIMAL LIQUID MEDIUM SUPPLEMENTED WITH VARIOUS AMINO ACIDS

Conidia of the indicated histidine-requiring mutants were inoculated into one ml of liquid minimal media. The concentrations of the supplemented amino acids are 10 mg/100 ml histidine and 50 mg/100 ml of the other amino acids.

Amino Acid Supplement	H3(K34)	H3(K26)	H3(K12)	H6
none	-	-	-	-
hist	+	+	+	+
hist + arg	+	+	+	+
hist + lys	+	+		
hist + ala	+	+		
hist + phenylala	+	+		
hist + meth	+	+	+	+
hist + arg + ala	-	-		
hist + arg + phenylala	-	-		
hist + arg + meth	-	-	-	-
hist + lys + ala	-	-		
hist + lys + phenylala	-	-		
hist + lys + meth	-	-		
hist + lys + arg	+	+		
hist + ala + meth	+	+		
hist + ala + phenylala	+	+		
hist + meth + phenylala	+	+		

when any one of the neutrals or any one of the basic amino acids is added, or when any two neutral or any two basic amino acids are added. The conclusion drawn from these data is that in the presence of neutral and basic amino acids the histidine entry sites are unavailable to histidine, whereas no concentration of neutral or basic amino acids alone prevents entry of histidine, indicating the presence of at least one available entry site when only neutral or only basic amino acids are present.

In summary, 1) the detailed kinetic analyses of uptake inhibition in Hymenolepis pointing to two entry sites for histidine, 2) the work correlating inhibition of growth of amino acid-requiring mutants with the inhibition of uptake of the same amino acids in wild type conidia, 3) the work showing the correlation between inhibition of growth of histidine-requiring mutants and the inhibition of histidine accumulation in wild type mycelium, and 4) the data in Table XI favor the conclusion that in Neurospora histidine uptake occurs through at least two entry sites, one a neutral amino acid transport-mediating site and the other a basic amino acid transport-mediating site.

ii) Genetic Analyses: If transport across membranes is a function either of specific carrier molecules or of the protein subunits of the membrane it is reasonable to expect that genetic information of the cell codes the transport specificity. It is impossible to test this hypothesis with organisms in which the genetics are unknown and in which the genetic background permitting observation of the desired mutations is missing or unavailable. Neurospora

crassa is an ideal genetic tool for such studies.

Histidine-requiring mutants provide a mirror of histidine uptake by growth alone. Inhibition is measured by the absence of growth or very reduced growth. Thus, if histidine enters the mycelia via two entry sites, one of which is inhibited by neutral amino acids and one by basic amino acids, a genetic mutation whose effect is to prevent histidine uptake through one of the sites would be characterized by the ability of the amino acids from the family whose entry site is not mutant to completely inhibit histidine uptake. From Table XI it can be seen that both a neutral and a basic amino acid must be present to completely inhibit histidine uptake in Neurospora with unimpaired uptake mechanisms. Two types of mutations are predicted, one a histidine-requiring mutant whose growth in histidine-supplemented medium is inhibited by basic amino acids alone, and one inhibited by neutral amino acids alone.

From a total of 2,000 single colony isolates (see Experimental Procedures, above), seven mutants with the predicted phenotypes were recovered. Six of the mutants are not inhibited by arginine but are inhibited by methionine, and one mutant is not inhibited by methionine but is inhibited by arginine. TableXII shows the growth inhibition patterns of these mutants in the presence of other inhibitor amino acids. It is clear from these data that the H34 and the H26II mutants are missing the basic amino acid transport-mediating system (they are inhibited by alanine and methionine), and mutant H26I is missing the neutral amino acid transport-mediating system (it is inhibited by arginine and lysine).

TABLE XII

GROWTH INHIBITION BY VARIOUS AMINO ACIDS OF THE TRANSPORT MUTANTS IN HISTIDINE SUPPLEMENTED MEDIUM

Amino Acid Supplement	Mutants						
	A34I	A34II	A34III	A34IV	A34V	A26II	A26I
lys	+	+	+	+	+	+	-
arg	+	+	+	+	+	+	-
ala	-	-	-	-	-	-	+
meth	-	-	-	-	-	-	+
arg + ala	-	-	-	-	-	-	-
arg + meth	-	-	-	-	-	-	-
lys + ala	-	-	-	-	-	-	-
lys + meth	-	-	-	-	-	-	-

The classical test for genetic involvement in altered phenotypes is the location of the cognate gene(s) within the genome. A second test for genetic identity is that of complementation, a test that can be made only with organisms capable of forming heterocaryons. One of the mutants was genetically mapped and tested for allelism with the other six.

Mutant A34IIIa was crossed to 19 morphological mutants (A), which represent at least two locations on each of the seven linkage groups of Neurospora. Of the 19 crosses, 4 were infertile and 10 yielded morphological mutant progeny that could not be distinguished from the transport mutants in wild type background. Fortunately, the five remaining crosses gave positive results, i.e., the transport mutation is heritable as a single gene mutation, and the transport mutation is linked to two mutations on the right arm of linkage group V. The transport mutation is clearly not linked to mutations representing three other linkage groups (Table XIII).

These crosses represent a more complicated analysis than is required for routine genetic mapping. For example, the parental train carrying the transport mutation (tpt^-) is also a histidine-requiring (his^-) mutant. Before locating the tpt gene, the probability of its being linked with his was 0.143, and the probability of its being sufficiently close to his to measure the linkage was much less. This means that tpt and his will combine with the morphological mutation introduced by the other parent with a greater than 50 per cent probability $1/7^{th}$ of the time. Since we now know that his and tpt are not linked we know that in only 25 per cent of the

TABLE XIII

ANALYSES OF FIVE CROSSES BETWEEN THE TRANSPORT MUTANT, A34IIIa, AND VARIOUS GENETIC MARKERS

Cross	Linkage Group of Marker	Total		Morphol. Markers		his⁻ Morphol. Markers*	
		Wild Type	Morphol. Marker	his⁺	his⁻	tpt⁻	tpt⁺
A34IIIa x yloA	IVL	65	72	33	39	20	19
A34IIIa x albA	IR	36	51	36	15	8	7
fla x A34IIIA	IIR	115	64	31	33	16	17
bis x A34IIIA	VR	100	90	36	54	20	34
spa x A34IIIA	VR	64	63	35	28	7	21

* tpt⁻ mutants were characterized by their ability to grow on histidine plus arginine, and their inability to grow on histidine + methionine and on histidine + methionine + arginine. tpt⁺ mutants were able to grow on the first two types of media but not on the third.

desired morphologically marked progeny will both his and tpt be introduced; i.e., not more than 1/4 of the progeny will show the desired genotype, tpt$^-$, his$^-$, morph$^-$. If the morphological marker and his are located on the same linkage group, the probability of recovering the desired phenotype decreases. The same is true if tpt and the morphological marker are linked. So, in all crosses a sufficient number of ascospores were isolated to permit sufficiently large numbers of the desired genotypes to appear. The protocol involved the isolation of 100 or more viable ascospores, the subsequent separation of the morphological progeny (about 1/2), and the determination, in this group, of the number of his$^-$ morphological types, and finally of the frequency of tpt$^-$ in the histidine-requiring morphologicals. When the frequency is about 50 per cent it is considered that tpt and the morphological marker are not linked; when the frequency is not the same, linkage varies with the degree of frequency difference.

Another aspect which made the analyses difficult is the identification of the tpt$^-$ morphological mutants. The tpt gene had to accompany his$^-$, and the identification is inhibition by methionine alone. Many of the morphological-histidine mutants are slow-growing and difficult to score, demanding, in all cases, at least three independent tests of each isolate.

Table XIII presents the data proving the linkage of A34III to sp (spray) and bi (biscuit) on the right arm of linkage group V. Also, it is obvious that tpt is not linked to the other three genetic markers. (The reason for the small number of his-alb progeny is

that his is linked to alb.) The calculated map distances between tpt and sp and between tpt and bi are 25 and 37 respectively.

The seven mutants were tested for allelism by the complementation test. The complementation test is possible with Neurospora since it is possible for nuclei of different genetic constitution to reside within the same cytoplasm. If the nuclei are mutant in exactly the same way one expects that they are unable to complement the other's deficiencies, whereas, if the two mutations involve different genes, then one nucleus compensates for the other, and vice versa. For example, two mutations, one unable to synthesize arginine and the other unable to synthesize methionine, can be shown to be non allelic by virtue of the fact that mycelia containing both nuclei grow on unsupplemented media.

Conidia from each of the seven tpt mutants were superimposed in all possible pairwise combinations on the following kinds of supplemented agar plates: histidine, histidine + arginine, histidine + methionine, histidine + arginine + methionine. The data in Table XIV show clearly that mutant A26I is non allelic to the others and that the others are allelic among themselves. This finding supports the conclusions derived from the growth experiments, i.e., the A34 and A26II mutants represent one transport-mediating site mutation (basic) and A26II represents the other (neutral); clearly two genetic loci controlling transport have been isolated.

In addition to the transport mutants reported here in which one or the other histidine entry site is mutant for histidine entry, several mutants were isolated that are capable of growth in the presence of histidine supplemented with excess arginine and methionine.

TABLE XIV

COMPLEMENTATION RESULTS OF SEVEN TRANSPORT MUTANTS TESTED IN ALL PAIR-COMBINATIONS ON AGAR MEDIA SUPPLEMENTED WITH HISTIDINE AND METHIONINE*

Mutants	Mutants**						
	1	2	3	4	5	6	7
1	-	-	-	-	-	+	-
2	-	-	-	-	-	+	-
3	-	-	-	-	-	+	-
4	-	-	-	-	-	+	-
5	-	-	-	-	-	+	-
6	+	+	+	+	+	+	+
7	-	-	-	-	-	+	-

* The complementation tests were done on four different agar media, histidine, histidine + arginine, histidine + methionine, and histidine + arginine + methionine. All colonies grew on histidine, mutants 1, 2, 3, 4, 5, and 7 grew on histidine + arginine, and none grew on histidine + arginine + methionine, making it impossible to score for heterocaryon growth. On histidine + methionine, however, mutant 6 grew sufficiently slow to permit effective scoring of heterocaryon growth.

** The mutant codes are as follows: 1 = A34I, 2 = A34II, 3 = A34III, 4 = A34IV, 5 = A34V, 6 = A26I, and 7 = A26II.

Thus far these mutants have not been analyzed either for uptake kinetics or genetically, but they may represent a mutation of either the basic or neutral amino acid entry site making it insensitive to the inhibitor amino acid. Plans have been made to further characterize these mutants.

iii) Conclusions: The present study of histidine uptake in Neurospora differs from more conventional approaches to this vital problem in that the actual measurements of uptake are less sophisticated, but the mechanisms of uptake are made a bit clearer. True, growth of a histidine-requiring mutant is not a direct measure of uptake, but both logic and the parallel between the results and those obtained by direct measure argue for the validity of such a measure of uptake. But, more important is the insight gained into the mechanism of amino acid entry through membranes.

For one thing, it has been conclusively demonstrated that entry is in some way a product of nuclear genes. The meaning of this becomes significant when considered against the backdrop of other experiments involving membranes. Membranes of mitochrondria have been isolated from a number of organisms, including Neurospora, and the evidence is compelling that the structural protein of mitochrondrial membrane (MSP) is composed of subunits of about 22,000 molecular weight and that the DNA coding the information of MSP is mitochrondrial in origin (41). By the same token, enzymes are known to be associated with MSP, and the genetic code for these enzymes is nuclear DNA (13). The suggestion has been made (41, 21) that mitochrondrial DNA codes all membrane structural protein, and evidence

is available that structural proteins from a wide variety of membranes are similar in size and composition (41). If this is true, the tpt mutations do not represent mutations in membrane structural protein, and it permits speculation to the effect that tpt genes code for "carrier protein," proteins that may be associated with plasma membranes in much the same way "enzyme assemblies" are associated with mitochrondrial membranes. Before this suggestion is put to the experimental test, however, an assay for the carrier protein must be developed. From that point, if such molecules do in fact exist, the same approaches that have been applied to enzymes will be possible, i.e., the determination of the primary structure of the protein, the elucidation of the active site, and the relationship of both to the cognate cistron (as has been done with tryptophane synthetase by Yanofsky and his coworkers, 45). In any event, it is clear that the first steps have been made, that is the isolation of mutants whose phenotypes are altered amino acid uptake, and whose general properties fit the notion that entry sites exist in membranes whose division is best described by placing the amino acids into families based on their general chemical properties. In some, as yet unknown, way two of these entry sites have been mutated, and the entry site mutations correlated with mutations in the nuclear DNA. If these mutations follow the generalization that mutations of DNA are expressed in specific proteins of the cell, it is very likely that mutant proteins (carrier proteins?) will be isolated. Once the site of mutation within the membrane is identified, the questions relating to carrier proteins may be answerable.

Prior to the identification of mutant proteins, however, it may be possible to determine whether the mutant basic amino acid entry site, for example, is unable to transport basic amino acids in general, or whether the mutation altered only its ability to transport histidine. The preliminary tests conducted thus far do not speak conclusively to this question, but evidence indicates that the site is mutant for transport of all normally recognized amino acids. Extension of the same question leads one to ask whether the mutant site recognizes amino acids of other families, whether mutations can be obtained that permit open entry, as opposed to closed entry, as observed here.

The validity of the claims that multiple entry sites with overlapping affinities are chemically separate, e.g., Christenson's alanine and leucine-preferring sites, must await tests in organisms permitting of genetic analysis. Even Christenson's kinetic data are much less definitive than analogous kinetic data obtained using Hymenolepis since he found overlapping affinities in all directions; this is not the case for histidine uptake in Hymenolepis. In view of this it seems reasonable that chemical individuality of uptake systems demands the more decisive approach afforded by the organisms used in these studies.

SUMMARY

1. Evidence is presented that in Hymenolepis diminuta and Neurospora crassa histidine uptake is mediated by both basic and neutral amino acid transport-mediating systems.

2. Histidine efflux in Hymenolepis is shown to be stimulated by external histidine, arginine or phenylalanine, all of which act on the outer surface of the membrane barrier.

3. Many transport-altered mutants of Neurospora were isolated and characterized, among which were: Six mutants in which histidine uptake can no longer occur through a basic amino acid entry site, and one mutant in which histidine uptake can no longer occur through a neutral amino acid entry site, and three mutants permitting uninhibited histidine uptake.

4. One of the mutants with an altered basic amino acid entry site was mapped, the genetic locus, called tpt, being located on the right arm of linkage group V.

5. Complementation analyses of the 6 basic amino acid and 1 neutral amino acid site mutants revealed that the 6 are allelic with each other and non-allelic with the seventh mutant, i.e., at least two distinct genetic loci were identified and found to control amino acid uptake.

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