Phospholipid Metabolism during Amino Acid Transport in Hamster Small Intestine* (33535)

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Although many characteristcs of transport systems have been described, the actual chemical identification of a carrier or a physical-chemical change in the state of the membrane has not been established.

The possibility that phospholipids may play an active part in the transport process has been considered for several reasons: (i) the concept that the carrier itself is lipid or lipid soluble, (ii) the universal association of phospholipids with animal cell membranes, making up 20–30% by weight of all animal cell membranes examined (1), (iii) the unique physical-chemical characteristics of phospholipids, existing in aqueous solutions in one of two forms, i.e., a lamellar structure as in the case of lecithin and sphingomyelin or a spherical micelle as in the case of the acidic phospholipids and lysolecithin (2).

Kavanau has postulated that a lamellar to micellar shift in membrane phospholipids may play a role in membrane functions including the transport process (3).

In addition, Rodbell (4) and Blecher (5) have demonstrated the ability of phospholipase C and phospholipase A to enhance glucose and amino acid transport in the rat epididymal fat pad, and have suggested that a lamellar–micellar shift of phospholipids in the membrane may provide a mechanism for the enhanced transport observed with these hydrolytic enzymes. If a lamellar–micellar shift in the major phospholipid of the cell membrane, lecithin, was operative during transport, one might observe enhanced incorporation of labeled fatty acid into lecithin during transport. Phospholipid metabolism during transport in the hamster small intestine was investigated to evaluate the possible role of such a phosphoglyceride cycle in the transport process. The results of this study demonstrate an increase in corporation of linoleic acid into lecithin at the site of most active amino acid transport in the hamster small intestine. This increased incorporation of fatty acid is apparently not related to *de novo* synthesis.

Materials and Methods. The entire length of the small intestine of the male golden hamster was divided into 3 equal parts and the middle and distal segments were used in making the everted intestinal loops. The intestinal loops were fashioned from 10–12 cm of small intestine by a minor modification of the method of Wilson and Wiseman (6). In each experiment the middle loop of one animal was compared with the middle loop of a second animal and similarly for the distal loops. Metabolic differences along the length of the small intestine did not permit comparison of the middle and distal loops from the same animal. The methods for loop preparation, recovery of serosal fluid (SF) and measurement of radioactivity were reported previously (7). The ^{32}P was added in tracer amounts as H_3PO_4 Radioactivity of the samples of serosal fluid before and after incubation were measured to determine the net increase in amino acid radioactivity.

Following drainage of the serosal fluid, the everted loop was placed on a glass rod and using a glass slide the mucosal layer was removed from the loop by gentle scraping and homogenized in 5 ml of acidified 0.05 *M* saline. An aliquot was removed and analyzed for protein according to the method of Lowry (8). The mucosal homogenate was then extracted with chloroform–methanol 2:1 (v/v) and the chloroform phase was dried under nitrogen and analyzed for lipid fractions using thin-layer chromatography and the ra-

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dioactivity of the lipid fractions were measured in the same manner, both as described previously (9).

Studies evaluating the effect of inhibition of fatty acid oxidation on transport were carried out using tissue slices in an attempt to assess the role of fatty acid as a possible substrate source for cell energy during transport. The middle one-third of the hamster small intestine was divided into segments and two or three randomly selected pieces of intestine were placed in an Erlenmeyer flask containing 5 ml of Krebs–Ringer phosphate buffer, (KRP) pH 7.4 (50–75 mg of tissue/flask). Flasks were gassed with oxygen during the 45 min of whole incubation. In order to allow movement of the inhibitor into the tissue prior to transport, the tissue was incubated for 15 min at 37° in the presence of 4-pentenoic acid. The control flasks contained only buffer. Following completion of the initial 15-min incubation, the tissue of both control flask and 4-pentenoic acid containing flasks were then transferred to transport flasks containing cycloleucine-(1–^{14}C) (4 mmoles, 900,000 cpm). The second incubation period was 30 min. Experimental and control flasks were run in duplicate. In selected experiments, duplicate flasks gassed with nitrogen were incubated similarly. In others, Na butyrate and glucose were added to the incubating media for both the 15- and 30-min incubation periods. Determination of transport in tissue slices was reported previously (7).

Oxidation studies were carried out by measuring the conversion of palmitate-(1–^{14}C) and D-glucose-(6–^{14}C) to $^{14}CO_2$ according to the method of Snyder and Godfrey (10). The mucosal homogenate was prepared by using a glass slide to scrape intestinal mucosa from the middle third of the hamster small intestine into oxygenated KRP buffer, pH 7.4. The mucosa was homogenized and 1-ml aliquots were removed and injected into the prepared oxidation flasks to start the reaction. An additional aliquot was removed for protein measurement (8). Incubations were carried out for 30 min and the reactions were terminated by the injection of 0.2 ml of 4 *N* H_2SO_4 into the reaction media. The collection of $^{14}CO_2$ for subsequent liquid scintillation counting was according to Synder and Godfrey (10).

TABLE I. Incorporation of Linoleate-1-^{14}C into Lecithin of Intestinal Mucosa during Leucine Transport.[a]

Middle segment (cpm/mg of protein)		Net transport (μmoles/ml/45 min)
NT	T	
1748	11,032	8.4
8156	9981	14.8
3660	16,453	10.0
5776	11,611	6.0
6234	9376	
5056	11,871	
Mean 5270 ± 2000	11,720 ± 2200	

[a] Control flask (NT) and transport flask (T) contained 5 ml of calcium- and glucose-free saline–Tris buffer (see text), pH 7.4, and 25 mμmoles of albumin-bound linoleate-1-^{14}C (800,000 cpm). Transport flask in addition contained leucine-4,5-^{3}H 12 mmoles (900,000 cpm). Incubation of the everted loops was carried out for 45 min at 37°.

Calculation of results. Amino acid transport in the everted loop was determined by measuring net increase in radioactivity in the serosal fluid following completion of incubation and dividing the cpm by the specific activity of the original serosal fluid to determine the net transport of amino acid in μmoles/ml in 45 min. Incorporation of ^{32}P and linoleic-(1–^{14}C) acid into the lipid fractions were expressed as counts per minute per milligram of tissue protein. Distribution ratios for transport in tissue segments were calculated employing previously determined measurements for total tissue water and extracellular fluid space (7).

Results. Linoleate incorporation. In experiments in which leucine transport was studied, incorporation of linoleic-(1–^{14}C) acid into lecithin was consistently greater in the middle transport loop as compared to the middle control loop (Table I). This difference was less marked in phosphatidyl ethanolamine and was absent in lysolecithin, sphingomyelin, phosphatidyl serine + phosphatidyl inos-

TABLE II. Incorporation of Linoleic-1-^{14}C Acid into Lecithin of Intestinal Mucosa during Cycloleucine Transport.[a]

Middle segment (cpm/mg of protein)		Net transport
NT	T	(μmoles/ml/45 min)
6530	9990	13.8
7660	12,920	9.2
5520	10,230	12.2
Mean 6500 $\pm$ 1000	11,000 $\pm$ 1600	

[a] Conditions for these experiments were identical to those in Table I except that cycloleucine-^{14}C 12 mmoles (900,000 cpm) was substituted for leucine-^{3}H in the transport flask.

itol as well as the triglyceride fraction. In the distal 1/3 of the intestine incorporation of linoleic acid into the various phospholipid fractions was not altered by the presence of amino acid transport. This may be related to the more heterogeneous cell population in the mucosa of the distal small intestine. Whereas the actively transporting epithelial cell comprises 80–90% of the cells of the mucosa in the midsmall intestine, it represents a much smaller fraction in the distal mammalian small intestine (11). In these experiments, transport of leucine was consistently greater in the middle segment and incoporation of fatty acid into lecithin was increased at the site of most active leucine transport.

In order to evaluate the role of leucine transport as opposed to the effect of intracellular metabolism of leucine on linoleic acid incorporation, three experiments were carried out using cycloleucine (α-aminocyclopentane-1-carboxylic acid) a nonmetabolizable amino acid. The middle segment again demonstrated increased incorporation of linoleic acid into lecithin in the transport loop as compared to the control loop (Table II).

Similar experiments using a basic amino acid, lysine, were carried out. Incorporation of linoleic acid into lecithin as well as the other phospholipids was not influenced by the presence or absence of lysine transport in either middle or distal loops of intestine. The reason for this lack of difference with lysine, whether due to the small transport rate or a unique difference in the transport mechanism for the basic amino acid is not discernible from these studies.

Phosphorus incorporation. Incorporation of ^{32}P into lecithin at 15, 30, and 45 min was not increased in the transport loop and similar findings were obtained for the other phospholipid fractions. If the increased incorporation of fatty acid into lecithin during transport had represented *de novo* synthesis, one would have anticipated increased incorporation of radioactive phosphorus into lecithin.

Transport inhibition. The ability of 4-pentenoic acid, a known hypoglycemic agent (12), to inhibit palmitate oxidation can be seen in Table III. In contrast, oxidation of D-glucose-(6–^{14}C) to $^{14}CO_2$ is not apparently influenced by 4-pentenoic acid. The capacity of 4-pentenoic acid to inhibit long-chain fatty acid oxidation while not affecting short-chain fatty acids has also been demonstrated in liver homogenates of the mouse (12). Incubation of tissue segments with increasing

TABLE III. Oxidation of Palmitate-1-^{14}C and D-Glucose-6-^{14}C to $^{14}CO_2$ in Presence of 4-Pentenoic Acid.[a]

Palmitate-^{14}C ($m\mu$moles)	($m\mu$mole/mg of protein) Control	4-Pentenoic	D-Glucose-UL-^{14}C (μmoles)	($m\mu$mole/mg of protein) Control	4-Pentenoic
10	0.91	0.39	2	1.75	1.80
50	0.72	0.39	2	1.83	2.10

[a] Reaction flask contained either 10 $m\mu$moles of palmitate-1-^{14}C (23,000 cpm/$m\mu$mole), 50 $m\mu$moles of palmitate-1-^{14}C (2700/$m\mu$mole) or 2 μmoles of D-glucose-6-^{14}C (121,000/μmole), and 1.5 mg of protein in calcium-free KRP buffer, pH 7.4. Incubations were carried out at 37° for 30 min. The 4-pentenoic flask contained in addition, 12 μmoles of 4-pentenoic acid.

TABLE IV. Inhibition of Cycloleucine-^{14}C Transport by 4-Pentenoic Acid in Fasted and Fed Animals.[a]

Conc. pentenoic acid (mM)	Distribution ratio; 4-Pentenoic		(μmoles/ml of intracellular fluid) 4-Pentenoic	
	Fasted	Fed	Fasted	Fed
0	5.75	4.03	18.2	11.9
6	3.20	3.85	9.8	11.3
12	2.95	2.71	8.4	7.0
18	2.40	2.00	5.3	4.1

[a] Reaction flask contained 5 ml of calcium- and glucose-free KRP buffer, pH 7.4, 4 mmoles of cycloleucine (900,000 cpm). The 4-pentenoic flask contained either 6, 12, or 18 μmoles/ml as indicated. Measurement of transport capacity was by distribution ratio and net increase in amino acid in μmoles per gram of tissue water above that obtained in nitrogen gassed flask. Incubations were carried out as described in the text.

concentrations of 4-pentenoic acid resulted in progressively smaller distribution ratios (Table IV).

In order to determine if incubation of tissue with 4-pentenoic acid resulted in nonspecific toxicity to the epithelial cell, experiments were carried out in which glucose or Na butyrate were added to incubation flasks in an attempt to reverse the effect of 4-pentenoic acid on transport by providing another substrate for energy production. In support of the observation that glucose oxidation by intestinal mucosa homogenates was unaffected by 4-pentenoic acid, the addition of glucose (5 mM) resulted in partial or complete reversal of the 4-pentenoic acid inhibition of transport. The results of a typical study are shown in Table V. In another experiment, sodium butyrate was able to partially reverse the effect of 4-pentenoic acid on transport (Table V).

These results suggest that the oxidation of long-chain fatty acid provides at least part of the cellular energy required for transport in the hamster small intestine, both in the fasted and fed state.

Discussion. These *in vitro* experiments in the hamster small intestine demonstrate an increased incorporation of linoleic acid into lecithin at the site of most active amino acid transport, i.e., the middle one third. In addition, they suggest that the augmented incorporation of fatty acid into these phospholipid fractions may be related to this transport process, either through alterations in the physical-chemical state of the lipoprotein membrane, through energy production for amino acid transport or other undefined mechanism. Further support for the concept that the enchanced fatty acid incorporation is related to the transport process may be obtained from the results of incorporation of fatty acid found with transport of the nonmetabolizable amino acid cycloleucine. Moreover, inhibition of active transport by using potassium as the major cation (129 meq of K) in the buffer, obviated the difference between the two loops. The augmented incorpo-

TABLE V. Reversal of Transport Inhibition by Glucose and Sodium Butyrate.[a]

	Distribution ratio	(μmoles/ml) ICF		Distribution ratio	(μmoles/ml) ICF
Control	4.54	12.86	Control	3.57	9.16
Control + glucose	5.26	16.93	4-Pentenoic	1.38	2.0
4-Pentenoic	2.50	6.86	+ Na butyrate (5 mM)	2.12	4.60
4-Pentenoic + glucose	4.46	13.51	+ Na butyrate (15 mM)	2.34	5.50

[a] Reaction flask contained calcium-free KRP, pH 7.4, with 4 mmoles of cycloleucine (900,000 cpm). The 4-pentenoic flask contained 12 μmoles/ml 4-pentenoic acid. The incubations were carried out as described in the text. Measurement of transport ability was carried out as per Table IV.

ration of fatty acid into lecithin and the absence of such findings in the triglyceride fraction suggest that transport does not result in a generalized nonspecific increase in fatty acid esterification. The increased incorporation of fatty acid into lecithin does not appear to be due to *de novo* phospholipid synthesis as shown by experiments using ^{32}P.

Lecithin, the major membrane phospholipid, exists in a lamellar form at pH 7.4 (2), and deacylation of lecithin to lysolecithin by phospholipase A would result in the formation of wedge-shaped molecules which exist in aqueous solutions as spherical micelles. Reacylation of lysolecithin to lecithin would complete a diacyl–monoacyl phosphoglyceride cycle which would be capable of producing significant changes in the physical-chemical state of this membrane phospholipid. The physical-chemical change in the state of this membrane phospholipid could produce conformational changes in the carrier which might enchance transfer of a substrate into the cell.

It was repeatedly demonstrated that energy production via aerobic metabolism is necessary for concentrative transport of amino acid and sugars in the mammalian small intestine (13–15). Dickens and Weil-Melherbe (16) observed that rat intestinal mucosa has a low respiratory quotient which is not influenced by the addition of glucose to the media. These findings suggest that fatty acid oxidation may be a major substrate for cell respiration in the rat and hamster small intestine. In addition, Crane (17) reported that 85% inhibition of glycolysis using calcium- and magnesium-free media with 0.02 *M* Na fluoride resulted in only 15% reduction in transport in the hamster small intestine. Previous work by Neptune *et al.* (18) using rat diaphragm and Bressler and Friedberg (9) using heart mitochondria suggested that the phospholipid fatty acids are part of the labile fatty acid pool of the cell and may serve as a source of substrate for fatty acid oxidation. Enhanced incorporation of fatty acids into lecithin at the site of most active transport could be related to increased oxidation of fatty acids in this phospholipid fraction with transport.

The results of these *in vitro* experiments demonstrate the ability of 4-pentenoic acid to inhibit long-chain fatty acid oxidation while having no effect on glucose oxidation. The inhibition of transport by 4-pentenoic acid was demonstrable in fed as well as fasted animals and these findings suggest that fatty acid oxidation provides at least part of the cellular energy required for transport irrespective of the animals' nutritional state.

Removal of fatty acid from the lecithin molecule during a lamellar to micellar shift could result in this fatty acid entering the fatty acid pool of the cell. This fatty acid would then be available for activation to an acyl CoA which would then enter the mitochondria and undergo oxidation or through a membrane acyltransferase enzyme, reacylate lysolecithin to lecithin. The enchanced incorporation of linoleic acid into lecithin may also be due to other as yet undefined mechanisms.

Summary. Previous studies of phospholipid metabolism during active transport have used ^{32}P and ^{14}C glycerol as a measure of phospholipid metabolism. In this investigation linoleic-1–^{14}C–albumin and ^{32}P were used in studying phospholipid metabolism during amino acid transport in the hamster small intestine. The results demonstrate an augmented incorporation of linoleic-1–^{14}C acid into lecithin at the site of maximum amino acid transport. The enhanced incorporation of fatty acid into lecithin may be related to: (a) a diacyl–monoacyl phosphoglyceride cycle resulting in a lamellar–micellar shift in this membrane phospholipid associated with transport, and/or (b) enchanced phospholipid fatty acid oxidation occurring with transport, or (c) other undefined mechanisms.

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Effects of Transition Metals and of Metal-Binding Antihypertensive Agents on Tryptamine Oxidase and Dopa Decarboxylase* (33536)

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Several antihypertensive agents, thought to lower blood pressure by a direct effect on vascular smooth muscle, were also noted to have the capacity to bind transition and related metal ions. Included among the clinically useful antihypertensive agents were the substituted hydrazines, 1-hydrazinophthalazine (Apresoline) and 1,-4-dihydrazinophthalazine (Nepresol) (1), and nitroprusside (2). Clinically less effective agents included the simple inorganic ions, thiocyanate (3) and azide (4). Because of the association of antihypertensive and metal-binding effects, the antihypertensive potency of other compounds known to bind metals tightly was evaluated. This evaluation revealed that experimental hypertension in animals could be corrected by ethylenediaminetetraacetate (5) and certain mercaptans (6). The mechanism by which metal-binding agents act on vascular muscle, if that is actually the basis of their antihypertensive effect, is speculative. Many of the metals which they bind, however, are found in human tissues (7). The possibility that their activity may be related to the binding of a specific metal led us to examine the effect of these substances and of transition and the related subgroup II metal ions on the activity of two crude enzyme systems: tryptamine oxidase and dihydroxyphenylalanine (dopa) decarboxylase, both of which involve the metabolism of vasoactive amines.

Methods. A homogenate containing monoamine oxidase was prepared from fresh livers of guinea pigs, using an equal volume of ice cold 0.067 *M* sodium phosphate buffer (pH 7.4). The homogenate was centrifuged in the cold, the resulting supernatant was dialyzed twice in the cold with stirring for 5 hr against 10 vol of the same phosphate

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