Biochimica et Biophysica Acta, 599 (1980) 664—672

BBA 78794

A HYDROLASE-RELATED TRANSPORT SYSTEM IS NOT REQUIRED TO EXPLAIN THE INTESTINAL UPTAKE OF GLUCOSE LIBERATED FROM PHLORIZIN

DEAN A. WARDEN, FRANKLIN F. FANNIN, JAMES O. EVANS, DAN W. HANKE and DONALD F. DIEDRICH

Department of Pharmacology, University of Kentucky Medical Center, Lexington, KY 40536 (U.S.A.)

(Received September 5th, 1979)

Key words: Glucose uptake, Phlorizin; Hydrolase; Intestinal transport; Disaccharidase

Summary

The fate of [^{3}H]glucose released from a wide range of [^{3}H]phlorizin concentrations by phlorizin hydrolase has been studied under conditions where the Na^+-dependent glucose transport system in hamster intestine is profoundly inhibited by the glucoside. At 0.2—2.0 mM phlorizin, the [^{3}H]glucose uptake was a constant 11—12% of that generated by the enzyme and at the highest level, it was reduced to that of passive diffusion. Glucose liberated from 0.2 mM [^{3}H]phlorizin is accumulated at a rate nearly equal to that found for 0.2 mM [^{14}C]glucose when this free sugar uptake is measured in a medium containing 0.2 mM unlabeled phlorizin. Furthermore, without sodium, the accumulation rates of hydrolase-derived or exogenous glucose are both reduced to the rate of [^{14}C]mannitol. Our results indicate that the glucose released from phlorizin enters the tissue via the small fraction of the Na^+-dependent glucose carriers which escape phlorizin blockade together with a mannitol-like passive diffusion. It enjoys a kinetic advantage for tissue entry over free glucose in the medium by virtue of the position of the site where it is formed, i.e. inside the unstirred water layer and near normal entry portals. No special hydrolase-related transport system, like the one proposed for disaccharides, needs to be considered to account for our findings.

Introduction

In the preceding paper [1], we presented evidence that phlorizin hydrolase in the intestinal brush border shares the characteristics previously described for

the disaccharidase in this membrane [2—5]. The enzyme splits phlorizin to liberate glucose which is then assimilated by the intestinal epithelium in the same manner found for the hexoses formed from disaccharides. We studied the phlorizin hydrolase system under conditions where the normal Na^+-dependent glucose transporter is profoundly inhibited by the phlorizin simultaneously present as the hydrolase substrate. Our findings fully support the original work reported by Crane and his group [2,3] and Parsons and Pritchard [4] that the sugar released by membrane glycosidases experience a kinetic advantage for absorption by an efficient capture mechanism. Whereas our earlier experiments were performed at a single phlorizin concentration, we have now investigated the transport kinetics of the [^{3}H]glucose liberated from a wide concentration range of the tritiated phlorizin substrate. The glucose concentration which is generated adjacent to the membrane surface from these phlorizin levels was estimated by an indirect approach. Increasing amounts of [^{14}C]glucose were added to media containing constant levels of unlabeled phlorizin. We thereby hoped to create a condition which would mimic that existing in the membrane micro-environment when these same concentrations of [^{3}H]phlorizin are split.

Experiments were also performed in sodium-free medium and with [^{14}C]-mannitol to gain further insight into the transport mechanism. Our results lead us to question whether it is necessary to postulate a direct carrier-like activity for the brush border glycosidases in order to account for the kinetic advantage for transfer of the hexose released by phlorizin hydrolase or the disaccharides.

Methods

The procedures used to make our measurements have already been described [1]. All transport and hydrolytic rates are given as nmol · min^{-1} · mg^{-1} dry villi. For the preparation of sodium-free, Krebs-Ringer phosphate buffers, the corresponding potassium, lithium, and Tris chlorides and phosphates were used instead of sodium salts. For the mannitol buffer, all the sodium chloride was replaced by an equal osmolar level of mannitol, and potassium phosphates were used instead of sodium phosphates. The pH of all solutions was 7.4.

Results

[^{14}C]Glucose and [^{14}C]mannitol uptake by villi

We first established the characteristics of the in vitro villus method, by comparing the uptake of [^{14}C]glucose and [^{14}C]mannitol over a range of concentrations from 0.02 to 50 mM during 3-min incubations. The results of these control studies are displayed in Fig. 1. Free glucose uptake resembles that found in the many other in vitro intestinal preparations; its entry can be represented as the sum of a saturable carrier process and a non-saturable, passive diffusion. In agreement with the report from Crane's laboratory [6], about 30 mM glucose in the medium is sufficient to saturate the glucose transporter; the apparent K_m is 1.6 mM under these conditions. Curve A (Fig. 1) serves as a reminder that when glucose is present at levels above saturation, or when active transport is inhibited, the passive diffusional portion of the sugar transport process represents a major fraction of the total uptake. This compo-

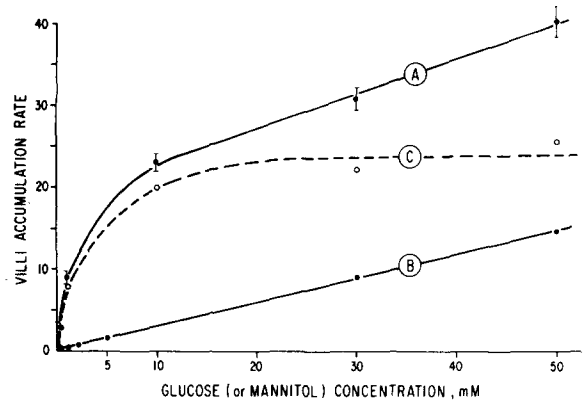

Fig. 1. Concentration dependent uptake by hamster intestinal villi of free glucose and mannitol added to the medium. Tissue incubation was in Krebs-Ringer phosphate buffer, pH 7.4, (3.0 ml) for 3 min, 37° C. The in vitro preparation and assay methods have been described [1]. Uptake rate is given as nmol · min^{-1} · mg^{-1} dry villi. Curve A: Uptake of total [^{14}C]glucose; metabolites (15% of the total ^{14}C in the tissue) are included since they represent glucose which was initially transported. Curve B: [^{14}C]mannitol uptake, representing the linear passive diffusion component of the process (slope = 0.29 nmol/min/mg villi/mM). Mannitol entered 20% of the calculated total villi space. Curve C: The saturable transport component of the total glucose uptake which is exposed by subtracting B from A. The apparent K_m and V for the carrier-mediated transport are 1.6 mM and 25.2 nmol/min.

nent could be easily underestimated especially when free glucose is generated directly at the cell surface where its concentration must surely be greater than that in the bulk medium.

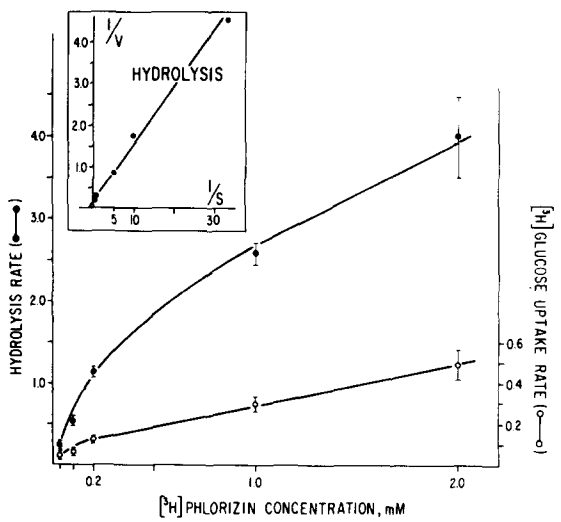

Fig. 2. Hydrolysis of phlorizin by phlorizin hydrolase and the villi accumulation rate of the liberated glucose. Substrate was [^{3}H]phlorizin, labeled with tritium only in the glucose moiety. Hydrolysis and accumulation rate (nmol · min^{-1} · mg^{-1} dry villi) are given as the means ± S.E. of 6, 18, 75, 18, and 6 determinations at phlorizin concentrations of 0.03, 0.1, 0.2, 1.0 and 2.0 mM, respectively. The extent of phlorizin hydrolysis during the 3-min incubation from its lowest to highest concentration was 11.8, 7.6, 8.3, 3.9 and 3.4%, respectively. In the inset, the hydrolysis rate data are presented as a double reciprocal plot. The apparent K_m for phlorizin hydrolysis is 1.1 mM and V is 6.6 nmol · min^{-1} · mg^{-1}.

Hydrolysis of [^{3}H]phlorizin and the uptake of the released [^{3}H]glucose

The liberation of [^{3}H]glucose from tritiated phlorizin by phlorizin hydrolase is shown in Fig. 2. It is likely that the hydrolytic rate was constant during the experimental period since only about 10% or less of the substrate was consumed. Some of the hexose released by the enzyme is accumulated by the tissue. This uptake also appears to be a combination of two mechanisms: a linear, non-saturable process plus a saturable component. At the highest phlorizin levels, a constant 11--12% of the glucose made available enters the tissue by a mannitol-like leak. At the low phlorizin concentrations (0.1 and 0.03 mM), transport efficiency was increased to 13.2 and 20.4% respectively which demonstrates that at these lower inhibitor levels, the Na^+-dependent phlorizin-sensitive glucose transporter is progressively less inhibited and assumes a proportionately greater role in the uptake process. When phlorizin is greater than 1 mM, however, less than 1% of carrier capacity remains. How then does the enzyme-generated glucose enter the tissue? Two mechanisms are possible: (1) the hydrolase directly transfers a constant 10% of the hexose into the cell interior as part of the cleavage reaction * or (2) the glycosidase-generated hexose enters via a passive, non-saturable, mannitol-type diffusion process through membrane 'pores' or 'channels'. In order to test the plausibility of this second mechanism, we performed the following experiments.

Villi accumulation of [^{14}C]glucose, [^{14}C]mannitol and the [^{3}H]glucose derived from [^{3}H]phlorizin in the absence of sodium

If our reasoning is correct then replacement of sodium in the medium should reduce the villi accumulation rate of [^{3}H]glucose to that found for [^{14}C]-mannitol. We therefore tested the effect of sodium depletion, first on the free glucose and mannitol uptake processes and then on the phlorizin hydrolase-related system.

The data assimilated in Table I indicate that the Na^+-dependent glucose carrier is functional in our preparation; the uptake rate of free [^{14}C]glucose, at 0.2 mM in Na^+-containing Krebs-Ringer phosphate buffer, is greater than 10 times that supported by the next best replacement ion, Li^+. Even lower transport rates were found with Tris and mannitol media. When the K^+ buffer was used, glucose uptake rate fell to that found for [^{14}C]mannitol control. In the presence of 0.2 mM unlabeled phlorizin and in Na^+ buffer, [^{14}C]glucose transport is inhibited more than 95%, but it is still twice the rate found for [^{14}C]mannitol under identical conditions. However, when Na^+ is omitted and replaced by Tris or mannitol, this residual carrier capacity is lost and the glucose uptake rate is then reduced to the passive, [^{14}C]mannitol control rate; Li^+, as expected [7], partially supported carrier mediated transport. Complete substitution by K^+ had the effect of reducing this sugar entry rate to a value which was less than the control [^{14}C]mannitol uptake rate but equal to that for [^{14}C]mannitol in K^+ buffer.

---

* If this were an enzyme-catalyzed process, then transport efficiency would be expected to decrease as saturating concentrations of phlorizin are reached. The glucoside's limited solubility prevented us from performing this crucial test. On the other hand, two populations of phlorizin hydrolase might exist: 88—90% of them have only cleavage capacity while the remainder act in a tightly-coupled, hydrolysis/transport manner.

TABLE I

EFFECTS OF SODIUM REPLACEMENT IN THE MEDIUM UPON PHLORIZIN HYDROLASE AND THE UPTAKE OF SUGARS IN THE VILLI

The mean ± S.E. rate values are given in nmol · min^{-1} · mg^{-1}. Number of experiments given in parentheses. Regardless of the medium used, from 24 to 35% of the liberated [^{3}H]glucose accumulated by the villi was in the form of metabolites. The accumulation rate of 0.2 mM [^{14}C]mannitol in the absence of phlorizin (data not shown) did not differ significantly from that found with the inhibitor present.

Permeant	Inhibitor	Villi accumulation rate				
		Control Na^+ (18)	Na^+ replacement in the Krebs-Ringer phosphate medium by			
			Li^+ (6)	$Tris^+$ (12)	Mannitol (11)	K^+ (6)
[^{14}C]Glucose (0.2 mM)	None	2.74 ± 0.14	0.233 ± 0.015	0.131 ± 0.005	0.068 ± 0.002	0.046 ± 0.003
[^{14}C]Glucose (0.2 mM)	Phlorizin (0.2 mM)	0.101 ± 0.007	0.073 ± 0.002	0.054 ± 0.005	0.049 ± 0.001	0.030 ± 0.002
[^{14}C]Mannitol (0.2 mM)	Phlorizin (0.2 mM)	0.048 ± 0.003	0.043 ± 0.002	0.042 ± 0.003	—	0.031 ± 0.003
[^{3}H]Glucose (? mM)	[^{3}H]-Phlorizin (0.2 mM)	0.118 ± 0.006	0.100 ± 0.006	0.028 ± 0.002	0.060 ± 0.005	0.048 ± 0.003
Hydrolysis rate of [^{3}H]Phlorizin (0.2 mM)		1.30 ± 0.05	0.952 ± 0.047	0.251 ± 0.024	0.807 ± 0.092	0.726 ± 0.108
Transport efficiency of the liberated glucose (in percent)		9.1 ± 0.4 *	10.6 ± 0.6	11.8 ± 1.3	7.6 ± 0.8	7.0 ± 0.5

* This value is not statistically different ($P > 0.05$) from the 11% found for 75 previous measurements [1].

Tests with [^{3}H]phlorizin confirm the observation of Malathi and Crane [8] that phlorizin hydrolase activity is optimal in the presence of Na^+. Our results indicate (Table I) that Li^+ is a better replacement to support hydrolytic activity than either mannitol or K^+ and we confirm previous reports [9,10] that substitution of Na^+ with Tris causes a profound decrease in phlorizin hydrolysis rate.

What are the effects of Na^+ omission on the accumulation of [^{3}H]glucose generated from phlorizin? The substitution of Na^+ by mannitol or K^+ leads to a 50% loss of this transport rate which is the expected decrease if, as we reasoned earlier [1], the Na^+-dependent glucose carriers that remain active in the presence of 0.2 mM phlorizin account for half of the total [^{3}H]glucose uptake under these conditions. Since Tris inhibited [^{3}H]phlorizin hydrolysis by 80%, it was not surprising to observe the lowest transport rate of [^{3}H]glucose in this buffer. Yet, substituting Tris for Na^+ caused a far greater reduction in hydrolysis than uptake and the best transport efficiency occurred in this medium. This may be the result of releasing glucose carriers from phlorizin blockade since the high affinity binding of the glycoside to the intestinal transporter is Na^+ dependent [11]. On the other hand, the result is comparable to what Parsons and Pritchard [4] and Crane's group [12] also found for the phlorizin-free disaccharidase-related transport system; Tris decreased the rate of maltose and sucrose hydrolysis but the efficiency with which intestinal cells were able to capture the enzyme-generated hexoses was increased.

With the exception of the Tris results, the effects of Na^+ replacement on

[^{3}H]glucose uptake essentially mimic those found for [^{14}C]glucose transport in the presence of phlorizin. Our model would predict this if the crucial assumption is made that the [^{3}H]glucose concentration in the micro-environment of the brush border approaches the concentration of its source, [^{3}H]phlorizin. Certainly, 0.2 mM is the maximum attainable concentration of the [^{3}H]glucose and this is why most of our early control studies with free [^{14}C]glucose were conducted at this level. Our findings support our assumption: [^{3}H]glucose endogenously formed from 0.2 mM [^{3}H]phlorizin in the presence of Na^+ is accumulated at essentially the same rate as that found for 0.2 mM [^{14}C]glucose in medium containing Na^+ and 0.2 mM phlorizin. Furthermore, replacement of Na^+ by either mannitol or K^+ has similar effects on both systems (Table I); the accumulation rate of both exogenous [^{14}C]glucose and the hydrolase-derived hexose is reduced to the passive diffusion found for [^{14}C]mannitol. It is important to notice that this reduction occurs even though [^{3}H]phlorizin is still being hydrolyzed at about 15 times the uptake rate. Clearly, transport is inhibited much more than hydrolytic action in these two media, an effect which is reflected by the low transport efficiency values.

Estimation of the [^{3}H]glucose concentration at the membrane surface

A direct measure of the effective [^{3}H]glucose concentration at the membrane surface cannot be made, but we have shown that the amount which enters the tissue can be readily assayed. If we assume that the [^{3}H]glucose is available for uptake only by the same mechanisms used by exogenous [^{14}C]-glucose when phlorizin is present, then the uptake of [^{14}C]glucose can be used in estimating the concentration of [^{3}H]glucose. Standard curves, one for each phlorizin concentration, were constructed in which [^{14}C]glucose uptake rate was plotted for an appropriate range of free concentration in the medium (Fig. 3). The [^{3}H]glucose uptake rate was now matched with the villi uptake of

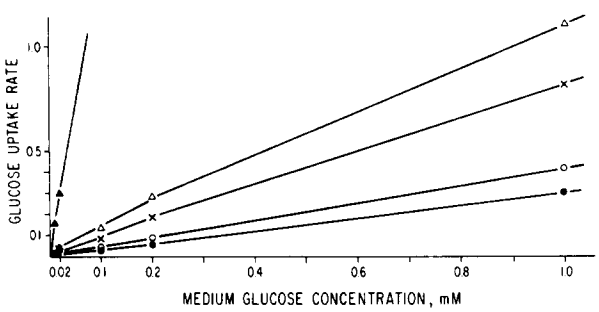

Fig. 3. Villi accumulation of [^{14}C]glucose in the presence of phlorizin. Each curve represents the rate (nmol · min^{-1} · mg^{-1} dry villi) of [^{14}C]glucose (plus metabolites) accumulated by villi during a 3-min incubation in normal Na^+-containing Krebs-Ringer phosphate buffer containing increasing amounts of unlabeled phlorizin. Phlorizin levels were 0.0 (▲), 0.1 (△), 0.2 (X), 1.0 (○) and 2 mM (●). The control uptake curve (no phlorizin) was constructed from previously reported data [1,13] and is the expanded early part of the curve shown in Fig. 1. As the inhibitor is increased to its solubility limits, the free glucose accumulation rate is progressively lowered; at 2.0 mM phlorizin, the glucose uptake curve has the same slope as mannitol uptake. Curves with phlorizin at lower levels show that there is residual Na^+-dependent carrier activity. Each point of the phlorizin-inhibited uptake curves represents the mean of 6—30 observations.

TABLE II

UPTAKE RATE OF LIBERATED [^{3}H]GLUCOSE COMPARED TO THAT OF [^{14}C]GLUCOSE IN THE PRESENCE OF VARIOUS PHLORIZIN CONCENTRATIONS

The phlorizin concentration represents either the unlabeled or the ^{3}H-labeled substrate/inhibitor. Its concentration remains essentially constant throughout the 3-min incubation inasmuch as less than 10% is hydrolyzed. The mean uptake rates ± S.E. in Column A are taken from the data in Fig. 2 (number of experiments given in parentheses). The values listed in Column B were obtained by finding the concentration of glucose on the abscissa of Fig. 3 which corresponds to the sugar uptake rate in Column A and using the proper phlorizin concentration curve.

Phlorizin (mM)	(A) Total uptake rate of [^{3}H]glucose released from phlorizin (nmol · min^{-1} · mg^{-1})	(B) Glucose concentration necessary to give the indicated rate in Column A (mM)
0.1	0.077 ± 0.01 (18)	0.06
0.2	0.133 ± 0.02 (75)	0.155
1.0	0.358 ± 0.08 (18)	0.82
2.0	0.492 ± 0.07 (6)	1.70

[^{14}C]glucose, and using the appropriate phlorizin standard curve, the sugar concentration in the medium (and presumably in the carrier environment) was estimated. Our underlying assumption requires that if this estimated [^{3}H]-glucose concentration exceeds the phlorizin concentration, then either (1) phlorizin hydrolase generates a higher concentration of free [^{3}H]glucose than the phlorizin substrate (an unlikely condition), or (2) the enzyme-liberated sugar enters the tissue by other than the known pathways, perhaps by a special hydrolase-related process. The estimated [^{3}H]glucose concentrations are shown in Table II. In every case, the glucose level which would support the transport rate was less than the phlorizin concentration. The findings are consistent with our hypothesis; phlorizin hydrolase-related transport involves the generation of free glucose directly at the membrane surface where it is delivered directly to the normal hexose transport sites at a greater concentration than can be achieved by random movement of free glucose from the bulk medium.

Discussion

The process by which glucose delivered from phlorizin is accumulated by intestinal mucosa may be viewed as a model of the disaccharidase-related-transport systems. Phlorizin hydrolase is intimately associated with lactase [14] and the latter enzyme confers the same kinetic advantage for transport to its hydrolysis products as sucrase and maltase [6]. Furthermore, the properties of the phlorizin hydrolase-linked sugar uptake system closely resemble those reported for the disaccharidases [1]. Using phlorizin as substrate to study the transport mechanism of glycosidase-derived glucose gives us an advantage not enjoyed by the workers who originally described the phenomena. The compound serves as both the glucose source and as a potent inhibitor of the well-known Na^+-dependent glucose transporter. To determine how the glycosidase-derived glucose is absorbed, it is important to know its effective concentration, not in the bulk medium, but directly at the membrane surface where the hydrolase generates it. Without this information, it is impossible to estimate

what fraction of the total sugar transport occurs via passive diffusion or the contribution of the glucose carrier system which escapes phlorizin inhibition. Crane and his colleagues have always stressed this point and their solution to this problem has been to compare the relative uptake rates of glucose generated by a disaccharidase with that formed by another brush border hydrolase, alkaline phosphatase [3,6,12]. At comparable hydrolytic rates, much more of the glucose liberated from sucrose than from glucose 1-phosphate was transferred through brush border vesicle membranes [15]. These results show that the mere generation of a high glucose concentration at the membrane is not sufficient to explain the preferential uptake of hexose from disaccharides; glucose generated by alkaline phosphatase does not experience this special transport process. Since all the evidence for a unique, direct transport role played by the disaccharidases focuses on this point, it is important to carefully consider whether phosphatase-derived glucose can serve as a reliable control for hydrolase-generated glucose at the membrane surface. We have no evidence to argue this point but have considered the following possibilities: (1) Glycosidases release their products in a compartment which is not shared by the glucose released by the phosphatase and from which there is greater access to normal sugar transport mechanisms. (2) Fewer copies of alkaline phosphatase exist per unit area of membrane than for the disaccharidases. Thus glucose from glycosides could be generated at higher density sites with better opportunity to use neighboring entry portals. (3) Glucose 1-phosphate is anionic and could experience distributional effects in the phosphatase microenvironment not applicable to uncharged glycosides. Furthermore, besides glucose, inorganic phosphate is generated in the reaction. The inorganic anion is also transported across the brush border membrane [16]; membrane potential could be affected which is known to influence glucose uptake [17,18].

Yet there are other arguments advanced by Crane and his colleagues to support their views and their work remains as strong evidence that the glucose from disaccharides enters the tissue by a different route than the Na^+-dependent glucose pathway and all diffusional pathways [2,3,6]. Furthermore, they have demonstrated this same phenomenon with brush border membrane vesicles in which the influence of unstirred layers are presumably at a minimum [15]. Our current studies with the phlorizin hydrolase system do not rule out their proposal that the disaccharidases play a direct role in the transport of split sugars. On the other hand, it is not necessary to view phlorizin hydrolase as a direct transferring agency in order to account for the kinetic uptake advantage of the glucose it liberates. Furthermore, at least two groups of clinical investigators have failed to find evidence for a special disaccharidase-related system in humans with glucose-galactose malabsorption [19,20], a syndrome in which, just as in our system, a functional Na^+-dependent sugar carrier is missing.

Acknowledgement

This work was supported by a National Institute of Health Grant 5 R01 AM06878.

References

1 Hanke, D.W., Warden, D.A., Evans, J.O., Fannin, F.F. and Diedrich, D.F. (1980) Biochim. Biophys. Acta 599, 652—663
2 Crane, R.K. (1975) in Intestinal Absorption and Malabsorption (Csaky, T.Z., ed.), pp. 127—141, Raven Press, New York
3 Crane, R.K. (1977) In Gastrointestinal Physiology, Int. Rev. Physiology II, Vol. 12 (Crane, R.K., ed.), pp. 325—365, University Park Press, Baltimore
4 Parsons, D.S. and Prichard, J.S. (1971) J. Physiol. 212, 299—319
5 Hamilton, J.D. and McMichael, H.B. (1968) Lancet ii, 154—157
6 Malathi, P., Ramaswamy, K., Caspary, W.F. and Crane, R.K. (1973) Biochim. Biophys. Acta 307, 613—622
7 Bihler, I. and Adamic, S. (1967) Biochim. Biophys. Acta 135, 466—474
8 Malathi, P. and Crane, R.K. (1969) Biochim. Biophys. Acta 173, 245—256
9 Kraml, J., Kolinska, J., Ellederová, D. and Hiršová, D. (1972) Biochim. Biophys. Acta 258, 520—530
10 Colombo, V., Lorenz-Meyer, H. and Semenza, G. (1973) Biochim. Biophys. Acta 327, 412—424
11 Tannenbaum, C., Toggenburger, G., Kessler, M., Rothstein, A. and Semenza, G. (1977) J. Supramol. Struct. 6, 519—533
12 Ramaswamy, K., Malathi, P., Caspary, W.F. and Crane, R.K. (1974) Biochim. Biophys. Acta 345, 39—48
13 Diedrich, D.F., Hanke, D.W. and Evans, J.O. (1975) in Intestinal Absorption and Malabsorption (Csaky, T.Z., ed.), pp. 143—153, Raven Press, New York
14 Leese, H.J. and Semenza, G. (1973) J. Biol. Chem. 248, 8170—8173
15 Ramaswamy, K., Malathi, P. and Crane, R.K. (1976) Biochem. Biophys. Res. Commun. 68, 162—168
16 Berner, W., Kinne, R. and Murer, H. (1976) Biochem. J. 160, 467—474
17 Toggenburger, G., Kessler, M., Rothstein, A., Semenza, G. and Tannenbaum, C. (1978) J. Membrane Biol. 40, 269—290
18 Geck, P. and Heinz, E. (1976) Biochim. Biophys. Acta 443, 49—53
19 Hughes, W.S. and Senior, J.R. (1975) Gastroenterology 68, 142—145
20 Fairclough, P.D., Clark, M.L., Dawson, A.M., Silk, D.B.A., Milla, P.J. and Harries, J.T. (1978) Pediat. 12, 1112—1114