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myo-Inositol transport in rat intestinal brush border membrane vesicles, and its inhibition by D-glucose

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The uptake of *myo*-inositol into rat intestinal brush border membrane vesicles (BBMV) has been investigated. It is demonstrated that *myo*-inositol is transported into the vesicles by a secondary active process, specifically using the sodium gradient as the driving force. In the absence of sodium gradient, the transport reaction is still sodium dependent, and rheogenic, indicating that a *myo*-inositol/sodium cotransport is likely to occur. A kinetic analysis shows an hyperbolic saturation process with a K_m of 0.16 ± 0.02 mM with respect to *myo*-inositol and V_{max} of 68.5 ± 21.2 pmol/min per mg protein. The transport is inhibited by D-glucose, phloridzin and few other sugars. The mechanism of D-glucose inhibition appears to be of the mixed type. Finally, the *myo*-inositol transport is trans-activated by *myo*-inositol itself, but not by D-glucose. It is concluded that *myo*-inositol is transported into rat intestine BBMV by a specific transport system, which is also able to bind D-glucose, but not efficiently transport it across the membrane.

Introduction

myo-Inositol is a cyclohexylic polyalcohol, that is considered an essential dietary factor not only in mammals [1] but also in birds and teleosts [1,2]. Deficiency syndromes have been described in some cases [2,3]. Its biosynthesis has been described in microorganisms and plants [4,5], and also in animal tissues, but at insufficient levels [6,7]. The biological importance of *myo*-inositol lies firstly in the fact that it is present in the structure of a class of membrane phospholipids, the phosphatidylinositols. Furthermore, some di- and triphosphate esters of *myo*-inositol, derived from the metabolism of phosphatidylinositols, have shown a hormone-like activity, as a second messenger in the cell cytoplasm.

Since *myo*-inositol is a vitamin for certain organisms, it is necessary for them to efficiently absorb the molecule in the intestinal tract, as well as equally efficiently reabsorb it in the renal tubule. In fact the uptake of *myo*-inositol by intestinal segments [8,9] and brush border membrane vesicles from rabbit kidney [10] and fish intestine [11] has been studied to some extent. From these studies with different organs from different sources a common feature emerges, i.e., the sodium dependence of the *myo*-inositol transport and the inhibitory interaction of D-glucose, some glucosides and other sugar related compounds with the transport process.

In this paper we have studied the transport of *myo*-inositol into brush border membrane vesicles from rat intestine, in order to characterize the transport process in this organ and to clarify its relationship with the process of glucose transport.

Materials and Methods

Chemicals

myo-[^{3}H]Inositol and D-[^{3}H]glucose were obtained from Amersham International (U.K.). All chemicals used were of analytical grade purity.

Preparation of membrane vesicles

Sprague-Dawley rats were killed by a blow on the neck. The intestines were immediately removed and placed in ice cold NaCl 0.9% solution. After being washed twice with the same solution, they were everted and scraped.

Brush border membrane vesicles (BBMV) were prepared within 3 h after death of the animal. Two to three intestines were used for the BBMV preparation. The scraping material was resuspended in 60 ml of ice-cold

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buffer containing 300 mM mannitol, 5 mM EGTA, 12 mM Tris (pH 7.1), then diluted 1/5 (v/v) with distilled water and homogenized with a mixer (Braun Melsungen, F.R.G.) for 2 min at maximum speed. Subsequently the BBMV were prepared according to the Mg^{2+}/EGTA method described by Hauser et al. [12], using a Sorvall RC 5B centrifuge equipped with an SS 34 rotor.

Enzyme assays

The purity of the BBMV preparations was monitored by measuring the specific activity of alkaline phosphatase (EC 3.1.3.1) according to Berner and Kinne [13].

The specific activity of alkaline phosphatase measured in the homogenate was 250 ± 70 nmoles of substrate consumed/min per mg protein, measured at 37°C, while in the final BBMV pellet the specific activity was 12(±1.8)-times greater than that measured in the homogenate (enrichment factor). Results are the mean ± S.D. of eight individual experiments.

Enrichment factors of marker enzyme activities of some contaminants were periodically measured and always found to be lower than 1. The markers used were: Na/K-ATPase for the baso-lateral plasma membrane; KCN-resistant NADH oxidoreductase, for endoplasmic reticulum; acid phosphatase for lysosomes [14].

Protein determination

Protein content was determined by the Bradford method [15], using the Bio-Rad kit (Bio-Rad, Richmond, CA, U.S.A.) and γ-globulin as a standard.

Uptake studies

Uptake studies were carried out using the rapid filtration technique as described elsewhere [16].

Briefly, 10 μl of membrane vesicles were mixed with 90 μl of incubation medium. At the time indicated, 20 μl of the mixture (equivalent to 100 μg of membrane protein) were directly pipetted onto the Millipore filter (45 μm pore size). The filter was immediately rinsed with 5 ml of ice-cold isotonic stop solution of the following composition: 150 mM NaCl, 2 mM Hepes-Tris (pH 7.5).

For kinetic studies the procedure was modified as follows. 10 μl of membrane protein (100 μg) were added to 20 μl of incubation medium. At the time indicated 1 ml of ice-cold stop solution was added to the incubation test tube, mixed on a vortex mixer and pipetted onto the filter. Thereafter 5 ml of stop solution were pipetted onto the filter to wash the aspecific radioactivity bound.

The radioactivity trapped on the filters was measured by standard liquid scintillation techniques. Membrane free incubation media were used as blanks; counts of the samples were at least 3-times higher than those of the blanks.

Each experiment was performed at least four times; only the results of a typical experiment are shown.

Where indicated parallel experiments, in the absence of sodium, were carried out to estimate the sodium-independent uptake and subtracted from the uptake measured in the presence of sodium.

Experiments were always performed in triplicate, data are generally expressed as pmol/mg protein per 30 s of incubation. Experimental scatter of triplicates was always less than 10% of the mean values reported.

In most experiments the incubation time was 30 s, which was sufficiently short to ensure that measurement took place during the linear part of the uptake. In fact, in control experiments in the presence of NaCl, the *myo*-inositol uptake was measured as a function of time at various substrate concentrations, in the range 0.025–2 mM. In the most unfavorable case (at the maximal concentration used) the uptake was linear up to 50 s (not shown).

The kinetics of the *myo*-inositol uptake was analyzed with the aid of a personal computer.

Where indicated, valinomycin in 95% ethanol was added to the membrane vesicles prior to incubation at 10 μg/mg protein. Ethanol did not exceed 1% (v/v) in the final incubation media and was added in control experiments at the same concentration.

Results

A first step in the characterization of the *myo*-inositol transport process across the brush border membrane vesicles has been to verify whether it is sodium dependent, and whether it uses the transmembrane sodium electrochemical gradient as a driving force for a secondary active transport process.

In Fig. 1 the time course of the *myo*-inositol transport into the brush border membrane vesicles from rat intestine is reported. In the absence of sodium, *myo*-inositol uptake into the vesicles occurs to a relatively low extent. In the presence of a 100 mM Na^+ gradient across the membrane, the uptake is much higher, and initially reaches levels higher than the equilibrium (overshoot), indicating a transient concentrative uptake and hence the presence of an active transport system. When the sodium gradient is abolished by loading vesicles with NaCl at the same concentration as that externally applied, the overshoot is not present but *myo*-inositol transport still occurs. The extent of the transport in these conditions is significantly higher than the sodium independent uptake.

The significance of this difference has been verified by evaluating the uptake at 30 s from four different experiments: the uptake in the presence of sodium was

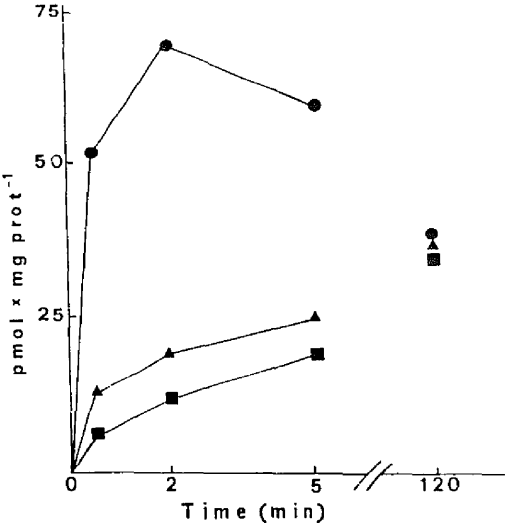

Fig. 1. Time course of the *myo*-inositol transport. A part of the membrane vesicles was suspended in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5), and incubated in media containing 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5) and 100 mM NaCl (●) or 100 mM KCl (■). A second aliquot of membrane vesicles was suspended in 100 mM mannitol, 100 mM NaCl, 20 mM Hepes-Tris (pH 7.5) and incubated in media of the same composition (▲). All incubation media contained 0.1 mM *myo*-[^{3}H]inositol.

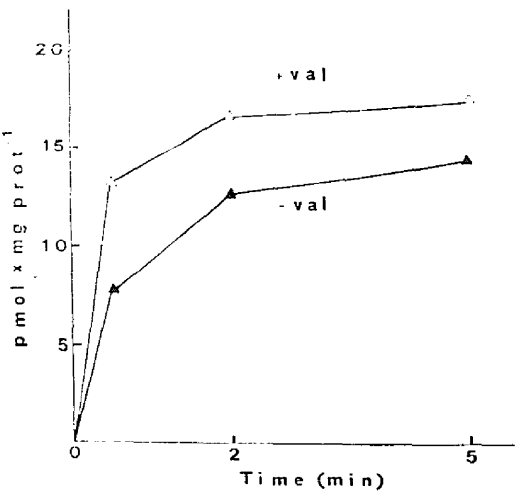

Fig. 2. Rheogenicity of the *myo*-inositol transport. Membrane vesicles, suspended in 100 mM mannitol, 100 mM NaCl, 100 mM KCl, 20 mM Hepes-Tris (pH 7.5) with (△) or without (▲) valinomycin, were incubated in 100 mM mannitol, 100 mM NaCl, 10 mM KCl, 20 mM Hepes-Tris (pH 7.5), 0.1 mM *myo*-[^{3}H]inositol (final external concentration). The data shown were subtracted for the sodium-independent uptake (see Methods).

found to be 103 ± 20% (mean value ± S.D.) higher than that in the absence of sodium.

These data indicate that the *myo*-inositol transport is sodium dependent and that the sodium gradient is used to take up *myo*-inositol against its concentration gradient. The effect of sodium is strictly specific. In fact none of the various cations tested (Table I) are able to substitute for Na^+ in stimulating *myo*-inositol transport.

In Fig. 2 the *myo*-inositol transport is measured in the presence of 100 mM NaCl equilibrated across the membrane, 100 mM KCl inside the vesicles and the ionophore valinomycin in the incubation medium. An electric potential, negative inside, is created by the movement of K^+ outside the membrane which is in turn generated by the specific ionophore. It can be observed that under these conditions the *myo*-inositol transport is increased as compared with the uptake measured in the absence of valinomycin. On the base of five different experiments, the increase at 30 s is significant, being 79 ± 18% (mean value ± S.D.). We can deduce that the transport is electrogenic, i.e., that positive charges move from outside to inside the vesicles. The obvious conclusion is that the system transporting *myo*-inositol operates a substrate/sodium cotransport, as do most of the carriers localized in the luminal plasma membrane.

A kinetic analysis of the *myo*-inositol transport as a function of the substrate concentration shows a Na-dependent process approaching saturation, in front of the linear trend of the Na-independent uptake, which appears as a diffusional process (Fig. 3A). The net sodium-dependent uptake, calculated as the difference between the two experimental curves, is reported in an Eadie-Hofstee plot (Fig. 3B). It apparently follows a Michaelis-Menten kinetics, with a V_{max} of 80 pmol/min per mg protein and a K_m of 0.15 mM. Mean values ± S.D., calculated from five different experiments, are: V_{max} 68.5 ± 21.2 pmol/min per mg protein and K_m 0.164 ± 0.019 mM.

TABLE I

Cation dependence of the myo-inositol uptake

Membrane vesicles, suspended in 300 mM mannitol, 20 mM Hepes-Tris (pH 7.5), were incubated in media containing 100 mM mannitol, 20 mM Hepes-Tris (pH 7.5), 0.1 mM *myo*-[^{3}H]inositol and 100 mM of various chloride salt indicated in the table.

Cations	pmol/mg protein per 30 s
Na^+	25.0
K^+	5.2
NH_4^+	5.7
Rb^+	5.1
Cs^+	5.0
Choline	4.5

TABLE II

Specificity of the myo-inositol transport: inhibition by glucosides

Membranes suspended in 300 mM mannitol 20 mM Hepes-Tris (pH 7.5), 100 mM KCl and valinomycin were incubated in media containing (final extravesicular concentrations): 100 mM mannitol, 100 mM KCl, 100 mM NaCl, 20 mM Hepes-Tris (pH 7.5), 0.1 mM *myo*-[^{3}H]inositol and 1 mM of the various glucosides indicated. The data are expressed as percentages of the control, after subtraction of the sodium independent uptake (see Methods).

Inhibitor (1 mM)	*myo*-[^{3}H]Inositol uptake (% of control)
Control	100
D-Glucose	54
L-Glucose	98
Phloridzin	17
D-Fructose	98
Methyl β-D-glucoside	67
L-Fucose	99
D-Xylose	97
3-*O*-Methylglucose	98

TABLE III

Uptake of myo-inositol and of D-glucose: reciprocal inhibition

Membrane vesicles, suspended in 300 mM mannitol, 100 mM KCl, 20 mM Hepes-Tris (pH 7.5) and valinomycin were incubated in media containing (final concentrations) 100 mM mannitol, 100 mM KCl, 100 mM NaCl, 20 mM Hepes-Tris (pH 7.5) and either 0.1 mM *myo*-[^{3}H]inositol (first column) or 0.1 mM D-[^{3}H]glucose (second column), plus the inhibitor as indicated. The data are expressed as percentages of the control, after subtraction of the sodium independent uptake (see Methods).

Inhibitor (mM)	*myo*-[^{3}H]Inositol (% of control)	D-[^{3}H]Glucose (% of control)
Control	100	100
Phloridzin (1)	19.5	4.0
myo-Inositol (10)	17.1	100
D-Glucose (10)	42.0	11.0

The ability of glucose and various glucosides to inhibit the transport of *myo*-inositol has been tested in experiments where the assayed compounds were present at the concentration of 1 mM, with respect to 0.1 mM concentration of *myo*-inositol. The results of a typical experiment are shown in Table II. D-Glucose gives an inhibition of about 50%, phloridzin over 80%. Among the other sugars tested, only methyl β-D-glucoside has an appreciable inhibitory effect.

The inhibitory effect of phloridzin, (a specific inhibitor of the glucose carrier), together with that of D-glucose, could lead to the interpretation that the transport of *myo*-inositol is mediated by the glucose carrier.

In this context, it is important to point out that the K_m for *myo*-inositol is of the same order of magnitude as that reported in the literature for the transport of glucose by its own specific carrier [17], while V_{max} is 2–3 orders of magnitude lower. Thus, if the transport of *myo*-inositol occurs by the D-glucose transport system, we have to expect a reciprocal inhibition by the two substrates. The ability of glucose and *myo*-inositol to inhibit each other has therefore been tested. It can be seen in Table III that, while 10 mM D-glucose gives a 60% inhibition on the transport of 0.1 mM *myo*-inositol, 10 mM *myo*-inositol has no effect on the transport of 0.1 mM D-glucose. In addition, 1 mM phloridzin

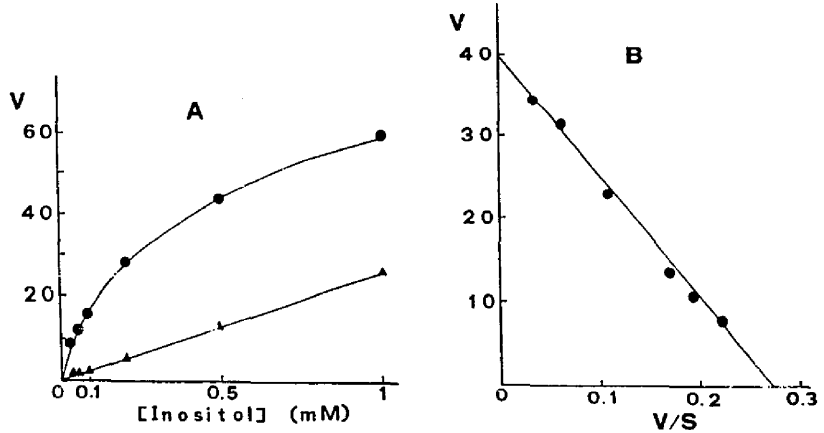

Fig. 3. Substrate concentration dependence of the *myo*-inositol transport. (A) Membrane vesicles were suspended in 300 mM mannitol, 100 mM KCl, 20 mM Hepes-Tris (pH 7.5) and valinomycin. The uptake at 30 s was started by adding the membrane vesicles to media containing 100 mM mannitol, 100 mM KCl, 20 mM Hepes-Tris (pH 7.5), *myo*-[^{3}H]inositol, at the concentrations indicated, and 100 mM NaCl (●) or 100 mM choline chloride (▲). (B) The Eadie-Hofstee plot has been drawn from the data in A, plotting the rate obtained in the presence of NaCl minus that in the presence of choline chloride, for each substrate concentration. V = pmol/mg protein per 30 s; V/S = μl/mg protein 30 s.

TABLE IV

Trans effect of myo-inositol and D-glucose on the myo-inositol uptake

Membrane vesicles were prepared in 300 mM mannitol, 100 mM NaCl, 20 mM Hepes-Tris (pH 7.5) (control) and in part preloaded with 1 mM *myo*-inositol or 1 mM D-glucose. One volume of each pool of vesicles was mixed with four volumes of the previous buffer containing in addition 0.2 mM *myo*-[^{3}H]inositol. The data shown were obtained by subtracting the sodium-independent uptake (see Methods).

in/out (mM)	*myo*-[^{3}H]Inositol (pmol/mg protein per 30 s)
- - Ino 0.2	8.1
Ino 1/ Ino 0.2	14.8
Gluc 1/ Ino 0.2 / Gluc 0.2	2.5
- -/ Ino 0.2 / Gluc 0.2	4.3

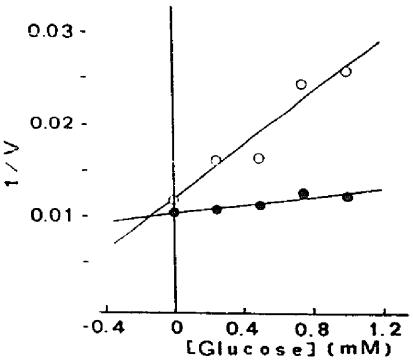

Fig. 5. Dixon plot of the glucose inhibition on the rate of *myo*-inositol transport. Experimental conditions as in Fig. 3, with *myo*-[^{3}H]inositol at the concentrations of 0.1 mM (○) and 0.3 mM (●) and glucose at the concentrations indicated. The data shown were obtained by subtracting the sodium-independent uptake (see Methods).

strongly inhibits both processes, but is less effective on the transport of *myo*-inositol.

The lack of effect of *myo*-inositol on the glucose transport, as well as the difference in the sensitivity to phloridzin of the two transport phenomena, leads to the conclusion that *myo*-inositol is not transported by the glucose carrier, but by another system that can be considered specific for it.

The mechanism of the glucose inhibition has been investigated by means of kinetic data.

A typical experiment showing the dependence of the transport rate on the substrate concentration, in the presence of 0.3 mM D-glucose, is reported in Fig. 4 as an Eadie-Hofstee plot. It is apparent, from the difference of the V_{max} values, that the inhibition is not of the competitive type. Although a number of experiments of this type were performed, some uncertainty

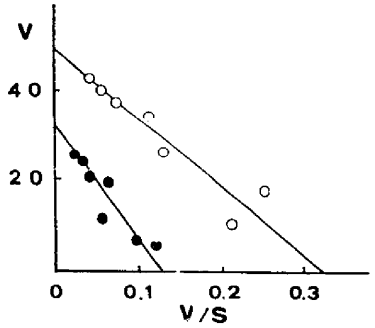

Fig. 4. Kinetics of the *myo*-inositol transport and its inhibition by D-glucose. The experimental conditions were as those in Fig. 3 in the control experiment (○). In addition the kinetics was measured in the presence of 0.3 mM D-glucose (●). The data shown as an Eadie-Hofstee plot were obtained by substracting the calculated experimental sodium-independent uptake (as in Fig. 3).

persists between a non-competitive and a mixed mechanism of inhibition. We have then measured the *myo*-inositol transport rate at various inhibitor concentrations. The results of this experiment are shown in Fig. 5 as a Dixon plot, where the reciprocal of *myo*-inositol transport rate is reported as a function of increasing concentrations of D-glucose. The two straight lines obtained at the two different substrate concentrations intersect at a point far from the abscissa, excluding the non-competitive inhibition with respect to a mixed mechanism. The K_i values for D-glucose, calculated from Eadie-Hofstee and Dixon experiments [18], are both in the range of 0.1–0.3 mM.

A further aspect of the transport mechanism that has been investigated is the effect of the presence of substrate or D-glucose inside the vesicles. In such experiments 100 mM NaCl was present both inside and outside the vesicles (Table IV). It can be seen that *myo*-inositol, at an internal concentration of 1 mM, strongly activates the uptake, while glucose under the same conditions has an inhibitory effect.

Discussion and Conclusions

The data presented in this paper demonstrate that, apart from an aspecific diffusional component, *myo*-inositol is taken up by isolated rat intestinal BBMV by means of a specific transport system. This transport system, like most of those occurring at the brush border level of the enterocytes, is sodium dependent and uses the sodium electrochemical gradient as the driving force. In fact, the electrogenicity of the system suggests that sodium is cotransported with the substrate. A sodium/*myo*-inositol cotransport system with similar characteristics has been already observed in hamster and

fowl intestinal segments [8,9], as well as in renal [10] and in fish [11] intestinal BBMV.

On the basis of the data of hyperbolic saturation (Fig. 3) and trans-stimulation (Table IV) by *myo*-inositol, a suitable carrier model can be hypothesised (with the assumption that, at the concentrations used, sodium saturates the carrier molecules, so that it does not limit the kinetics of the carrier *myo*-inositol interaction). The model involves a fast first step (the binding of the substrate [and Na^+] to the carrier), and a further slow process involving: (i) rearrangement of the substrate-carrier complex from the 'outside' to the 'inside' conformation, (ii) release of the substrate (and Na^+), and (iii) rearrangement of the free carrier molecule in the opposite direction [19]. The two rearrangement steps are the rate determining ones. In addition, the effect of trans-stimulation by *myo*-inositol observed (Table IV) indicates that the backward movement of the free carrier is slower than the forward movement of the carrier-substrate-Na complex.

As regards the interaction of glucose with the *myo*-inositol transport system, in renal BBMV a competitive inhibition, combined with an effect of dissipation of the membrane potential, has been suggested [10]. In intestinal segments and BBMV, however, a non-competitive mechanism of inhibition has been claimed by various authors, which has been explained in different ways [8,9,11]. From the present data the inhibition by glucose appears to be of the mixed type. In our experimental conditions dissipation of the sodium electrochemical gradient by the glucose transport can be excluded. In fact the inhibition of the *myo*-inositol transport has been observed both in the absence of membrane potential (clamped to zero by the presence of potassium and valinomycin), and in the absence of sodium chemical gradient (Table IV). Therefore the behaviour of D-glucose as a mixed inhibitor may simply indicate that its binding to the carrier molecule makes the binding of *myo*-inositol more difficult, and that the ternary complex formed is unable to rearrange itself to the opposite conformation. Furthermore, the lack of a trans-stimulation effect by D-glucose on the *myo*-inositol transport suggests that the glucose-Na-carrier complex is not more 'mobile' than the free carrier itself.

In conclusion, the data presented indicate, in agreement with other authors [8–11], that *myo*-inositol is transported across the brush border vesicle membrane by a specific carrier inhibitable by phloridzin and glucose, whereas *myo*-inositol is not able to interact with the glucose carrier. On the other hand, D-glucose interacts significantly with the *myo*-inositol carrier but is poorly, or not at all, transported by it. This last conclusion definitively rules out the possibility that the *myo*-inositol carrier is the same as the glucose carrier which, as reported [17], is subject to a strong trans-stimulation effect by D-glucose.

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References

1 Rose, R.C. (1981) in Physiology of the Gastrointestinal Tract (Johnson, L.R., ed.), pp. 1231–1242, Raven Press, New York.
2 Halver, J.E. (1969) in Fish in Research, pp. 209–257, Academic Press, New York.
3 Holub, B.J. (1982) in Advances in Nutritional Research (Draper, H.H., ed.), Vol. 4, pp. 107–141, Plenum Press, New York.
4 Chen, J.W. and Charalampous, F.C. (1963) Biochem. Biophys. Res. Comm. 12, 62–67.
5 Loewus, F.A. and Kelley, S. (1962) Biochem. Biophys. Res. Commun. 7, 204–208.
6 Eisenberg, F., Jr. (1967) J. Biol. Chem. 242, 1375–1382.
7 Withing, P.H., Palmano, K.P. and Hawthorne, J.N. (1979) Biochem. J. 179, 549–553.
8 Caspary, W.F. and Crane, R.K. (1970) Biochim. Biophys. Acta 203, 308–316.
9 Lerner, J. and Smagula, R.M. (1979) Comp. Biochem. Physiol. 62A, 939–945.
10 Hammermann, M.R., Sacktor, B. and Daughaday, W.H. (1980) Am. J. Physiol. 239, F113–F120.
11 Vilella, S., Reshkin, S.J., Storelli, C. and Ahearn, G.A. (1989) Am. J. Physiol. 256, G501–G508.
12 Hauser, H., Howell, K., Dawson, M.R.C. and Bowyer, D.E. (1980) Biochim. Biophys. Acta 602, 567–577.
13 Berner, W. and Kinne, R. (1976) Pflügers Arch. 361, 269–277.
14 Scalera, V., Storelli, C., Storelli-Joss, C., Haase, W. and Murer, H. (1980) Biochem. J. 186, 177–181.
15 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
16 Hopfer, U., Nelson, K., Perrotto, J. and Isselbacher, K.J. (1973) J. Biol. Chem. 248, 25–32.
17 Semenza, G., Kessler, M., Hosang, M., Weber. J. and Schmidt, U. (1984) Biochim. Biophys. Acta 779, 343–379.
18 Webb, J.L. (1963) Enzyme and Metabolic Inhibitors, Vol. I, Academic Press, New York and London.
19 Stein, W.D. (1989) Methods Enzymol. 171, 23–62.