concentrations which do not markedly affect the vitality of uninfected cells. The inhibition of virus growth is irreversible and takes place even when the drug treatment of cell cultures is restricted to 2 h, either before or after the infection period. No inhibition can be seen when the drug is allowed to act directly on virus particles before cell infection (Figure 1). Target of the pyrimidine action seems to be the assembly of virus particles, which is completely prevented, while the replication of infectious virus RNA and the net synthesis of virus proteins are scarcely affected as well the early virus-induced blockade of cell protein synthesis and the cytopathic effect (Figure 2). The pyrimidine analogue does not act on virus assembly directly, but rather by impairing the RNA coating ability of virus proteins made in its presence: when a 3-h period in drug-free medium is allowed to elapse between virus infection and drug treatment, proteins synthesized in that period assemble into complete, infectious virus, the RNA synthesized later in the presence of the drug (Figure 2). Researches are in progress to better define the intimate mechanism of action of 2-amino-4, 6-dichloropyrimidine, as well as the specificity of the antiviral effect[7].

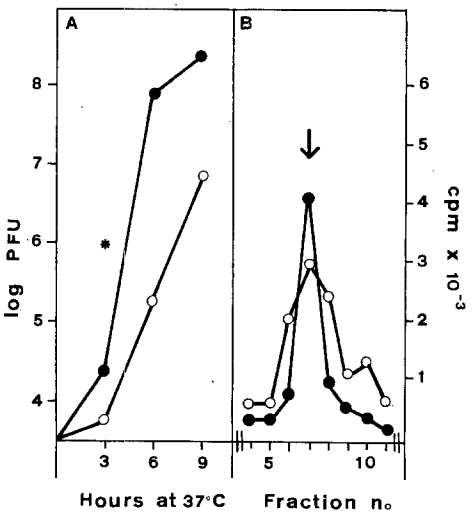

Fig. 3. Ability of virus proteins, synthesized before drug-treatment, to assemble into complete, infectious virus particles, RNA synthesized later in the presence of the drug. Infected cells were incubated for 3 h at 37 °C in Hank's BSS containing AMD 2 μg/ml, and then treated with the pyrimidine derivative. A) Production of infectious virus, titrated as PFU (○) and incorporation of ^{3}H-uridine, 0.3 μCi/ml from time 0 (●). B) Incorporation into virus particles of ^{3}H-leucine (5 μCi/ml) added to the medium from time 0 up to 3 h post-infection and of ^{3}H-uridine (3 μCi/ml) added to the medium 3 h after infection. Cells were harvested at the 8th h and treated as indicated in Figure 2. The asterisk in A) indicated the moment of drug addition to the culture. The arrow in B) indicates maximum infectivity in the sucrose gradient.

Riassunto. La 2-amino-4, 6-dicloropirimidina impedisce la formazione di proteine capsidiche capaci di organizzare con lo RNA virale particelle di poliovirus complete ed infettanti.

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STUDIORUM PROGRESSUS

Kinetics and Na-Dependence of Riboflavin Absorption by Intestine in vivo

Introduction. Intestinal absorption of riboflavin has been studied both in vivo and in vivtro. In vivo absorption was related to urinary excretion of the vitamin which had previously been administered to the digestive tract. Obviously this approach to the problem is insufficient, since there are too many, barriers interposed in between and thus one cannot really draw conclusions on the mechanism of absorption of the vitamin across the intestinal barrier. However Levy and Jusko[1] were the first to affirm, using this experimental model, that the absorption was saturable in fasting subjects. Using analogous techniques, Stripp[2] had already come to the same conclusions. Since then, both Mayersohn et al.[3] and Christensen[4] have confirmed these results in both man and rat.

The results of these studies in vivo are in contradiction with the earlier work of Spencer and Zamchek[5], of Spencer and Bow[6] and of Turner and Hughes[7] who measured the absorption of riboflavin, using the technique of the everted sac. These authors concluded that there was a passive diffusion of the vitamin across the intestinal wall; they also showed that the rate of diffusion of the vitamin was the same in either the mucosal-serosal or the serosal-mucosal direction. These results are surprising and are possibly influenced by the external conditions used in vitro.

The aim of this work is to study the intestinal absorption of riboflavin in vivo, while adopting an approach to the problem which is different to previous authors. The technique used was the perfusion of a fixed segment of intestine in vivo. Using this method the kinetics of absorption of the vitamin were studied as well as the role played by sodium during absorption by the intestinal tract.

Material and methods. 6-to-10-week-old wistar rats were used in all experiments. They were fasted 24 h before the experiment and Nembutal was used as a narcotic. After abdominal incision, a segment (6–8cm) of

[1] G. Levy and W. J. Jusko, J. Pharm. Sci. *55*, 285 (1966).
[2] B. Stripp, Acta pharmac. tox. *22*, 353 (1965).
[3] M. Mayersohn, S. Feldman and M. Gibaldi, J. Nutr. *98*, 288 (1969)
[4] S. Christensen, Acta pharmac. tox. *27*, 27 (1969).
[5] R. P. Spencer and N. Zamcheck, Gastroenterology *40*, 794 (1961).
[6] R. P. Spencer and T. M. Bow, J. nucl. Med. *5*, 251 (1964).
[7] J. B. Turner and D. B. Hughes, Q. Jl. exp. Physiol. *47*, 107 (1962).

Table I. Influence of NaCl on the intestinal absorption of water in rat in vivo

Group No.	Perfusion time (min)	Perfusion medium	Average rate of water absorption (mg/min/g tissue)	P value
1	15–45	NaCl	33 ± 6.6 (10)	> 0.05
	45–120	NaCl	30 ± 4.0 (10)	
2	15–45	NaCl	40 ± 6.5 (5)	< 0.001
	45–120	Mannitol	−2 ± 2.4 (5)	
3	30–60	NaCl	30 ± 5.5 (5)	< 0.05
	60–120	LiCl	13 ± 3.1 (5)	

The rat was perfused with a medium containing D-glucose (2 mg/ml), riboflavin (100 ng/ml) and either isotonic NaCl, isotonic mannitol, or isotonic LiCl. The results are given as means plus or minus the standard error of the mean (SEM). The number of experiments is given in brackets.

Table II. Intestinal absorption of hydrosoluble vitamins and of D-glucose: comparison between the different kinetics

Substrate	Concentrations (μM)	K_t (μM)	V_{max} (nmole/min/g wet weight)
Thiamin [a]	0.1 – 1.0	0.16	0.012
Pteroylglutamic acid [b]	0.1 – 1.0	0.7	0.07
Biotin [c]	100 – 2000	1000	17.9
Riboflavin [d]	0.27 – 2.7	3	0.26
D-glucose [d]			
'Carrier' 1	100 – 60000	700	1040
'Carrier' 2	100 – 60000	15000	1200

[a] VENTURA et al.[19]: in vitro experiments in rat.
[b] SMITH et al.[20]: in vitro experiments in rat.
[c] BERGER et al.[9]: in vitro experiments in hamster.
[b] HONEGGER and SEMENZA, to be published: in vitro experiments in hamster.

intestine, situated in the proximal jejunum region, was chosen. The loop of the intestine was perfused with the aid of peristaltic pump at rates of either 0.137 ml/min or 0.157 ml/min. At the end of the perfusion the segment was removed, washed in isotonic NaCl, dried on adsorbent paper and then weighed.

The rate of water absorption may be determined by two methods: 1. By the addition of tritiated inulin (1 g/100 ml) to the perfusion medium. This method was found however to be inexact because a relatively important fraction of the inulin is in fact absorbed. MILLER and SCHEDL[8] have recently shown that the absorption of inulin takes place in an analogous system: while perfusing the intestine with radioactive inulin, they have found radioactivity in both plasma and urine. In our laboratory, a second method for the measurement of water absorption has been used with more efficient results.

2. By weight, the flow into the loop (D_e) and the flow from the loop (D_s) are determined. The rate of absorption of substrate is calculated in the following formula:

$$\frac{(C_i \times D_e) - (C_f \times D_s)}{P}$$

where C_i and C_f are the concentrations of substrate in the perfusion medium before and after the perfusion across the intestinal loop. P is the wet weight of intestine perfused. The concentration of substrate in the perfusion medium is determined by measuring radioactivity. [$^{14}C_2$] D-riboflavin, [^{3}H] inulin and [$^3H_6$] D-glucose are counted in a liquid scintillation counter. To 100 µl of perfusion medium 10 ml of scintillator (POPOP 100 mg, PPO 10 g, toluol 2 l, éthanol 1.2 l) is added.

[8] D. L. MILLER and H. P. SCHEDL, Gastroenterology *62*, 48 (1972).

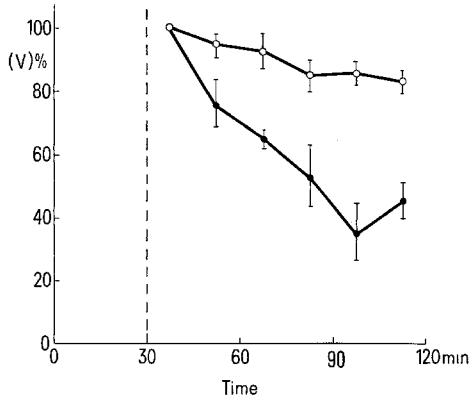

Fig. 1. The effect of NaCl on the intestinal absorption of riboflavin in the rat in vivo. ○, controls: rats perfused for 2 h with a medium containing isotonic NaCl, D-glucose (2 mg/ml) and riboflavin (100 ng/ml). $n = 10$; ●, rats perfused for 45 min with the above medium followed by a second medium containing isotonic mannitol, D-glucose (2 mg/ml) and riboflavin (100 ng/ml). $n = 5$; V, mean rate of absorption of riboflavin expressed as a percentage of the initial rate of absorption measured between the 30th and the 45th min; T, time of perfusion in min; the bars show the SEM. The effect of mannitol is statistically significant: $P < 0.001$ when the t-test is applied to values obtained between the 60th and 75th min of perfusion.

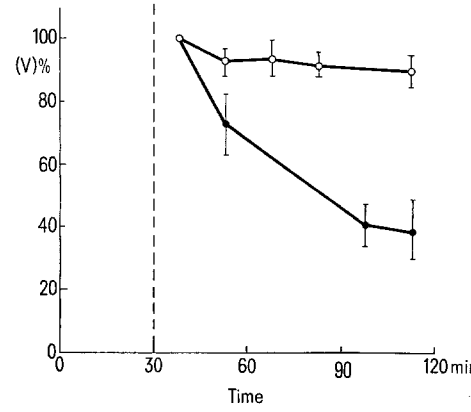

Fig. 2. The effect of NaCl on the intestinal absorption of D-glucose in the rat in vivo. Experimental conditions: see legend of Figure 1. ○, controls ($n = 9$); ●, rats perfused with isotonic mannitol (in place of NaCl) from the 45th min ($n = 5$); V, mean rate of absorption of D-glucose expressed as a percentage of the initial rate of absorption measured between the 30th and the 45th min; T, perfusion time in min; the bars show the SEM. The effect of mannitol is statistically significant: $P < 0.01$ when the t-test is applied to values obtained between the 90th and the 105th min of perfusion.

D-riboflavin an D-glucose were supplied by Fluka A.G., Switzerland. Fresh solutions of riboflavin (stored in darkness at 4°C) were prepared at least once a week. Inulin and D-mannitol are products of Merck (Darmstadt, Germany). Inulin was dissolved by heating and was conserved for not more than a few days in the cold. Dimethyl-POPOP and PPO were supplied by Packard Instrument Company, Inc., USA. [$^{14}C_2$] D-riboflavin (0.045 mg/ml), [$^3H_6$] D-glucose and [^3H] inulin were supplied by the radiochemical centre, Amersham, England. These radioactive chemicals were stored in aqueous solutions at –20°C.

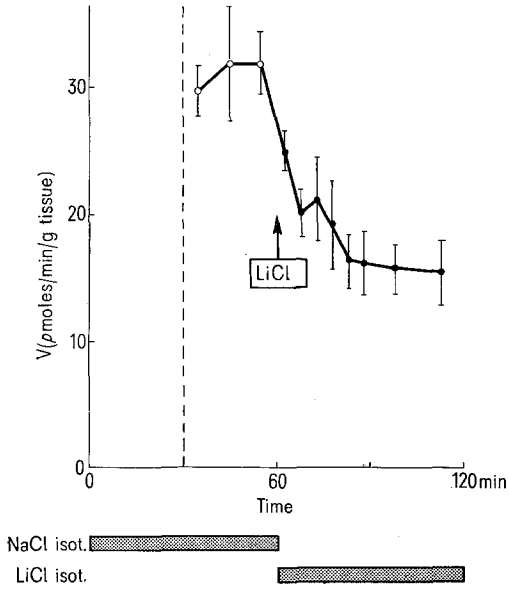

Fig. 3. The effect of Na^+ (replaced by Li^+) on the intestinal absorption of riboflavin in the rat in vivo. The rats are perfused for 1 h with a medium containing isotonic NaCl, D-glucose (2 mg/ml) and riboflavin (100 ng/ml) followed by a second medium containing isotonic LiCl (155 mM), D-glucose (2 mg/ml) and riboflavin (10 ng/ml). The rate of perfusion is 0.157 ml/min. ○, isotonic NaCl; ●, isotonic LiCl; T, time of perfusion in min; V, mean rate of absorption of riboflavin in $p$mole/min/g tissue. The bars show the SEM, $n = 5$. The effect of LiCl is statistically significant: $P < 0.005$ when the t-test is applied to values obtained between the 75th and 90th min of perfusion and when the group of rats pefused only with NaCl is taken as a control (see Figure 1).

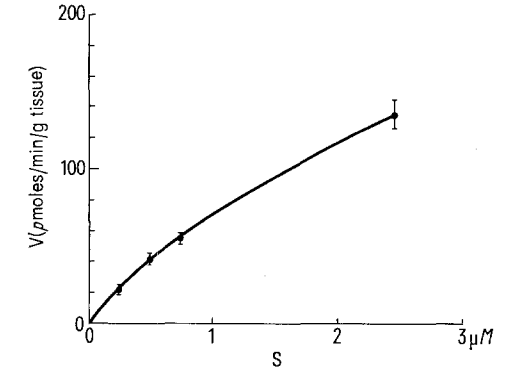

Fig. 4. Influence of the concentration on the rate of intestinal absorption of riboflavin in the rat in vivo. The rats are perfused for 150 min with a medium containing isotonic NaCl, D-glucose (2 mg/ml), and various concentrations of riboflavin. $n = 5$. The rate of perfusion is 0.137 ml/min. V, rate of absorption of riboflavin ($p$mole/min/g tissue); S, Concentration of riboflavin in the medium in μM. The bars show the SEM.

Results. The influence of NaCl on the intestinal absorption of riboflavin, D-glucose and water. The results presented in Figures 1 and 2 and Table I show that the absence of NaCl in the perfusion medium brings about an important decrease in the absorption of riboflavin and D-glucose. Even when the perfusion medium is kept isotonic with mannitol, in the absence of NaCl, there is a complete block of water absorption; at times there was even a secretion of water. The results obtained when NaCl is replaced by an equimolar concentration of LiCl are similar to those above (see Figure 4 and Table I). The influence of LiCl on the absorption of D-glucose was not determined in this set of experiments. It has also been noted that, from the time that NaCl is removed from the perfusion medium, there is an increase in the production of mucus by the tissue.

Kinetics of intestinal absorption of riboflavin in vivo. In the same rat the rates of absorption of different concentrations of riboflavin were measured. A group of 5 rats gave results presented in Figures 4 and 5. Figure 4 clearly shows a saturation in the absorption of the vitamin. The graphical representation of the Lineweaver-Burk plot (Figure 5) allows one to calculate the apparent K_t value (3 μM) and the V_{max} value (264 $p$moles/min/g tissue).

Discussion. In agreement with the work of LÉVY and JUSKO[1], it has been possible to show in this paper that the transport of riboflavin across the intestinal barrier is kinetically saturable. This would indicate that the absorption of riboflavin is a biological process (facilitated diffusion) and not a simple physico- chemical diffusion as proposed by certain authors. The K_t is of the order of 3 μM and the V_{max} of the order of 300 $p$moles/min/g tissue. BERGER et al.[9] have looked for a transport system having a very low K_t. The K_t of biotin, as measured in vitro, is only slightly lower than that of the sugar transport system, whereas the V_{max} is much lower. The other hydrosoluble vitamins have a very low K_t and V_{max} when compared to that of the sugar transport (see Table II).

MAYERSOHN and GIBALDI[10] have studied the effect of Na^+ on the intestinal absorption of riboflavin in an in vitro system. When replacing the Na^+ ion by K^+ or NH_4^+ they obtained a reduction of about 50% of riboflavin transport. However, the replacement of Na^+ by Li^+ or by $Tris^+$ did not bring about an inhibition in the absorption of riboflavin. This result is different from that reported in this work where Li^+ inhibits the absorption of riboflavin by about 50%. Elsewhere these authors have established a relationship between the intensity of inhibition of water and riboflavin transport. They conclude that the transport of the vitamin is not dependent on the presence of Na^+ in the incubation medium but is influenced by the content of water of the incubated tissue. In vivo it was noted that the absence of sodium in the perfusion medium brought about a decrease in the absorption of water, and it would seem doubtful that under these conditions there is an increase in the water content of these tissues. However, since this was not measured in vivo, one can only suggest that the swelling of the intestinal cells which takes place in the absence of Na^+ is due simply to the experimental conditions in vitro and that this is not the real cause of the inhibition of transport.

[9] E. BERGER, E. LONG and G. SEMENZA, Biochim. biophys. Acta *255*, 873 (1972).

[10] M. MAYERSOHN and M. GIBALDI, J. Pharm. Sci. *60*, 225 (1971).

The effects obtained by replacing NaCl by mannitol in the perfusion medium could be due to Mannitol in itself, to the absence of Na^+, to the absence of Cl^- or to a combination of the above. Mannitol is often used by different research workers to replace NaCl. It has never been reported that isotonic mannitol in itself had an effect on intestinal absorption. However, recently PONZ and LLUCH[11] noted that D-glucose absorption could be progressively inhibited by replacing NaCl by *Tris*Cl, KCl, LiCl reaching a maximum inhibition with mannitol. The effect can be reversed by replacement with NaCl; however, the reversal is much slower after treatment with mannitol. The authors conclude that there is a local effect with these replacement substances, the least effect being with *Tris*-Cl.

An essential role by Cl^- has never been attributed to absorption phenomena. This would seem to be confirmed by experiments presented in this work: both LiCl and mannitol decrease the intestinal absorption of riboflavin to the same extent.

Most research workers will admit that numerous cations, both organic and inorganic, are incapable of replacing Na^+ in certain transport processes, especially those which play a role in the transport of a substrate against an electrochemical gradient (SCHULTZ and CURRAN[12]). For the intestinal absorption in vivo, the role of luminal Na^+ is still controversial. This work shows that the presence of Na^+, in the perfusion medium of the intestinal lumen, is necessary for normal absorption of riboflavin or D-glucose.

A striking observation in these results is the total blockage of water absorption when NaCl is replaced by mannitol and a significant reduction when LiCl replaces NaCl. The relationship between the sodium pump and intestinal absorption of water is still under discussion (see review of this problem by MATTY and NOBLE[13]). Certain authors (SMYTH and TAYLOR[14]) have shown that in vitro the inhibitors of the sodium pump also inhibit water absorption. Other authors (MALAWER et al.[15]) however, affirm that in the jejunum of man the transfer of Na^+ is secondary to water absorption ('solvent drag'). FAELLI et al.[16] have shown, while perfusing an intestinal segment in vitro, that the replacement of NaCl by isotonic *Tris*-Cl, or that the addition of phlorizin to the incubation medium, brings about a decrease in the passage of water and D-glucose. It is not possible to say to what extent those phenomena are related and if the blockage of water absorption could have repercussions on the absorption of riboflavin or D-glucose. As regards this problem, FLESHLER and NELSON[17] have shown that, in the dog in vivo, even when there is a secretion of water by the intestine, the rate of absorption of L-alanine remains unchanged. It is thus possible that the absorption of water (as well as D-glucose or riboflavin) is stimulated by Na^+ present in the perfusion medium.

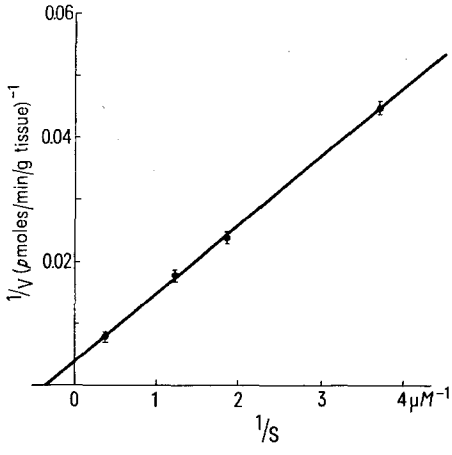

Fig. 5. Kinetics of intestinal absorption of riboflavin in vivo. Lineweaver-Burk plot. Experimental conditions see Figure 4. $K_t = 3\mu M$; $V_{max.} = 264$ *p*moles/min/g tissue.

Because of the decrease in intestinal absorption of riboflavin in the absence of Na^+, it is still difficult to speak of active transport of the vitamin against a concentration gradient in the intestinal mucosa. In fact FAELLI et al.[16] have shown that acetamide, which presumably diffuses passively across the intestinal barrier, is also sensitive, in vitro at least, to the absence of NaCl in the perfusion medium. BIHLER[18] has shown, based on in vitro studies, that only sugars which are not actively transported (e.g. mannose and arabinose) are not sensitive to the absence of Na^+ in the incubation medium. Further complementary studies are necessary to clarify this hypothesis.

Conclusion. The transport of riboflavin across the intestinal mucosa is certainly not by a system of passive diffusion. It necessitates the presence of a specific transporter in the membrane, thus explaining saturation kinetics noted. The transport is dependent on the presence of Na^+ in the perfusion medium. However, it is not as yet possible to say whether transport is against an electrochemical gradient or a system of facilitated diffusion with simple equilibrium of concentrations extra- and intra-cellular.

Résumé. L'absorption intestinale de la riboflavine est mesurée in vivo par perfusion d'un segment proximal du jéjunum chez le rat. Le transport de la vitamine à travers la muqueuse intestinale suit une cinétique de saturation ($K_t = 3\mu M$; $V_{max} = 0{,}26$ nMole par min et par g de tissu frais). Il est fortement réduit (de 40 à 50%) lorsqu'on substitue au NaCl isotonique du milieu de perfusion du mannitol ou du LiCl isotoniques.

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3 July 1973.

[11] F. PONZ and M. LLUCH, Rev. esp. Fisiol. *27*, 369 (1971).
[12] S. G. SCHULTZ and P. F. CURRAN, Physiol. Rev. *50*, 637 (1970).
[13] A. J. MATTY and H. M. NOBLE, Hormones *3*, 42 (1972).
[14] D. H. SMYTH and C. B. TAYLOR, J. Physiol., Lond. *136*, 632 (1957).
[15] S. J. MALAWER, M. EWTON, J. S. FORTRAN and F. J. INGERFINGER, J. clin. Invest. *44*, 1072 (1965).
[16] A. FAELLI, G. ESPOSITO, G. GAROTTA and V. CAPRARO, Experientia *27*, 652 (1971).
[17] B. FLESHLER and R. NELSON, Gut *11*, 240 (1970).
[18] I. BIHLER, Biochim. biophys. Acta *135*, 466 (1967).
[19] V. VENTURA, G. FERRARI, R. TAGLIABUE and G. RINDI, Life Sci. *8*, 699 (1969).
[20] M. E. SMITH, A. J. MATTY and J. A. BLAIR, Biochim. biophys. Acta *219*, 37 (1970).
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