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COMPARISON OF INTESTINAL PENETRATION OF CORTISOL AND CONVALLATOXIN: DEMONSTRATION OF A TRANSPORT MECHANISM FOR CARDIOTONIC STEROIDS*

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SUMMARY

1. The dependence of the penetration of cortisol and convallatoxin (strophanthidin-α-L-rhamnoside) from the site of administration (mucosal or serosal solution) and from the steroid concentration was studied in the perfused small intestine of the rat *in vitro* (method of FISHER AND PARSONS).

2. Within a concentration range of 5 to 400 μM, the penetration rate of cortisol is practically independent of the steroid concentration. The penetration from the perfusate into the serosal medium proceeds approximately three times more rapidly than in the reverse direction.

3. The penetration rate of convallatoxin is markedly dependent on the glycoside concentration. Increasing the convallatoxin concentration in the perfusate from 5 to 400 μM causes a 95 % decrease in penetration rate. The concentration dependence of the convallatoxin penetration from the serosal medium into the lumen is similar, but less pronounced. Therefore at a low glycoside administration on the serosal side, the penetration rate is smaller, but at a high concentration, it is ten times greater than in the normal direction.

4. When convallatoxin is introduced into the perfusate, the glycoside content of the mucosal section of intestinal tissue is three times greater than in the serosal section. When convallatoxin is dissolved in the serosal medium, no significant differences within the different tissue layers can be observed. Under the latter conditions, the glycoside content of the tissue shows only a slight increase after the first 15 min, while the glycoside penetration into the perfusion solution continues steadily.

5. Protein assays in solution and tissues have shown that the anomalous penetration behaviour of convallatoxin cannot be ascribed to a release of cell- or protein-bound glycoside.

6. A simple interpretation of these and previous results can be derived from the assumption that convallatoxin is absorbed by means of a transport mechanism with saturation kinetics (carrier transport). The reversal of the relationship of the penetration rates measured at high convallatoxin concentrations is thus explained by the

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saturation conditions of the transport mechanism on both sides of the luminal membrane of the mucosal cell. The relevance of a transport mechanism for cardiotonic steroids to the glycoside accumulation by the heart and to the theories of drug absorption is discussed.

INTRODUCTION

Previous investigations have demonstrated that the intestinal absorption of cardiotonic steroids cannot be exclusively explained in terms of free diffusion. The rate of intestinal absorption of polar cardiac glycosides is reduced both *in vivo*[1,2] and *in vitro*[1,3] with increasing drug concentration in the lumen. Furthermore, the absorption of the cardiac glycoside, convallatoxin, was found to be inhibited by cortisol in the perfused rat intestine *in vitro*[3] and to be dependent on the Na^+ concentration in the perfusate[4]. It was concluded from these results that the absorption of cardioactive steroids occurs by means of a hitherto undescribed transport mechanism of limited capacity (carrier mechanism). The present paper provides further evidence for the existence of this transport mechanism from a kinetic viewpoint; since the substrate concentration on the two sides of the membrane is known to influence penetration in distinctive fashions in cases of transport and diffusion[5], the two possibilities may be kinetically distinguished by a study of this nature.

Accordingly, small intestines of rats were artificially perfused using the apparatus of FISHER AND PARSONS[6], the cardiac glycoside convallatoxin (strophanthidin-α-L-rhamnoside) or cortisol (for comparative purposes) being dissolved at different concentration levels in either the luminal perfusate or the serosal solution. The movement of the drugs into the tissue or into the opposite solution under different conditions was determined.

MATERIALS AND METHODS

The experiments were generally carried out by the methods previously described[3,7]. In the following section, only the principle of the method and necessary additions are provided.

Chemicals

Convallatoxin was supplied by the chemical division of Dr. Madaus and Co., Köln-Merheim, cortisol USP XVI was purchased from Schering A.G., Berlin, and most other chemicals (of analytical grade) were obtained from E. Merck A.G., Darmstadt.

Animals and experimental techniques

Male Wistar rats with a body weight of 150–200 g from our own strain were employed for all experiments. The perfusion of a gut loop approx. 30 cm long was performed in the apparatus of FISHER AND PARSONS[6]. The outer surface of the intestine was bathed by a similarly oxygenated and thermostatically controlled solution. The volume of the perfusate was 40 ml, and that of the serosal solution 60 ml. The temperature was maintained at 37.5°, and the perfusion period lasted 15, 30 or 45 min.

Except for the addition of steroid, luminal and serosal solutions were identical and had the following composition (mM): 110.6 NaCl, 7.0 KCl, 3.0 $CaCl_2$, 1.0 $MgSO_4$, 0.9 sodium phosphate buffer (pH 7.4), and 27.8 glucose. Convallatoxin and cortisol could be dissolved in this medium without any solubiliser.

Determinations

At the end of the experiments, the serosal solutions (in the case of mucosal steroid supply) and the mucosal solutions (in the case of serosal steroid supply) were concentrated *in vacuo*. Cortisol was determined by the method of SILBER AND PORTER[8]. After extraction and chromatographic purification convallatoxin was estimated with alkaline picrate solution[9] by methods described previously[3]. To determine the glycoside content of the tissue, the gut loop was washed inside and outside with constant volumes of perfusate containing no convallatoxin, then dissected lengthwise and blotted gently with filter paper. Subsequently, the mucosa with adhering connective tissue was separated from the underlying muscular layers by means of the method of DICKENS AND WEIL-MALHERBE[10]. The resultant tissue components will be designated "mucosal section" and "serosal section" respectively. The two tissue sections were extracted by methods previously described, which included homogenisation, extraction with chloroform–ethanol mixtures, purification of the extracts by acetone precipitation, distribution between aqueous ethanol and light petroleum, and finally paper chromatography. Convallatoxin was estimated in the eluates from the paper chromatograms by the method of NEUWALD[9].

Biuret reagent was used to determine the protein content of trichloroacetic acid precipitates of aliquots of the mucosal and serosal solutions and of ethanolic homogenates of gut tissue. Labtrol "Totalprotein" (Asid-Institut, München) served as a standard.

Definitions and calculations

Since the term "absorption" is generally reserved for the flow of substances from the gut lumen to the blood (or the artificial bath), the term "penetration" is preferred, for the purposes of the present work, to refer to movement in both directions. The amounts of steroid determined in the perfusate or serosal solution after penetration through the gut wall were expressed per cm gut length and per h of perfusion time. To characterise the relationship between penetration and concentration of the drug in the medium, the expression "relative penetration" is introduced and defined as follows:

$$\text{relative penetration (penetration rate)} = \frac{\text{penetration/cm gut} \cdot \text{h}}{\text{concentration/ml fluid}} \times 100\,\%$$

When the uptake into the tissue is determined, this uptake is expressed per g tissue wet weight. All measurements are corrected for methodological losses and blank values by means of control experiments in the usual manner.

RESULTS

Penetration of cortisol

To study the characteristics of convallatoxin penetration when introduced on either side of an artificially perfused rat intestine, a direct comparison with a sub-

stance which penetrates by simple diffusion was considered necessary. Cortisol was chosen for this purpose (Fig. 1). When the steroid is only added to the perfusate, the relative penetration from a 5 μM solution was 6.64 $\pm$ 0.42 %, and from a 400 μM solution, 8.49 $\pm$ 0.59 %. An 80-fold increase in the drug concentration only resulted in a small and statistically insignificant rise in the rate of penetration. When the steroid is only added to the serosal solution, the relative penetration is lower than when the drug is dissolved in the mucosal solution, and the absolute values are again directly proportional to the substrate concentration in the serosal solution. The penetration rate is consequently once more practically independent of the drug concentration. The values of the relative penetration in this case are 2.73 $\pm$ 0.26 % from a 5 μM solution, and 2.15 $\pm$ 0.23 % from a 400 μM solution; the difference is again not statistically significant.

Penetration of convallatoxin

In contrast to the uptake of cortisol, the penetration rate of convallatoxin demonstrates a clear-cut dependence on the glycoside concentration (Fig. 2). When the convallatoxin concentration in the perfusate is increased from 5 μM to 400 μM, the relative penetration falls off from 3.80 $\pm$ 0.36 % to 0.175 $\pm$ 0.047 %, *i.e.* to approx. 5 % of the original value. The differences in relative penetration from a 400 μM solution and from a solution containing 25 μM substrate or less are significant at a level of $P < 0.0027$. When the glycoside is introduced into the serosal solution, one obtains a result that is surprising at first glance. When the convallatoxin concentration in the serosal medium is 400 μM, the relative penetration into the mucosal solution is 1.77 $\pm$ 0.01 %, that is to say, ten times greater than when the same concentration of the glycoside is introduced on the luminal face of the tissue. The penetration

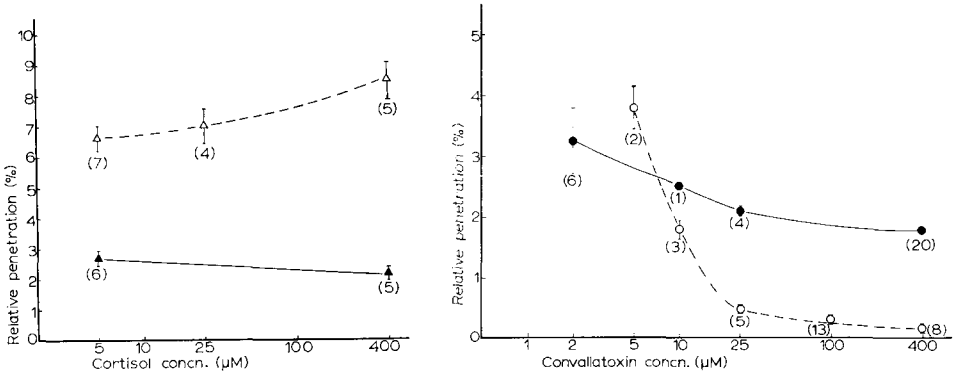

Fig. 1. Penetration of cortisol through the perfused small intestine of the rat *in vitro*. For the composition of media see METHODS; temperature 37.5°, time 45 min. Abscissa (logarithmic): cortisol concentration in perfusate and serosal solution respectively; ordinate: relative penetration into serosal solution and perfusate respectively. △---△, steroid supply from the mucosal side (perfusate); ▲—▲, steroid supply from the serosal side. The means $\pm$ S.E.M. are shown; the number of experiments below the points in parentheses.

Fig. 2. Penetration of convallatoxin through the perfused small intestine of the rat *in vitro*. Composition of media see METHODS; temperature 37.5°, time 45 min. Abscissa (logarthmic): convallatoxin concentration in perfusate and serosal solution respectively; ordinate: relative penetration into serosal solution and perfusate respectively. ○---○, glycoside supply from the mucosal side (perfusate); ●—●, glycoside supply from the serosal side. The means $\pm$ S.E.M. are shown; the number of experiments below the points in parentheses.

rate increases when the serosal concentration is decreased, but the increase proceeds more slowly than in the case of mucosal glycoside supply. Nevertheless, the difference between relative penetrations from serosal solutions of 400 μM and 25 μM or less is again significant at a level of $P < 0.0027$. The penetration-rate curves of serosal and mucosal supply of the drug bisect in the substrate concentration range of 5–10 μM (Fig. 2); at very low concentrations, convallatoxin is transported more rapidly from lumen to blood than in the opposite direction. These results confirm, as expected, that the penetration of convallatoxin, a substance presumed to be transported, is dependent on concentration and site of administration in a manner very different from that of cortisol which penetrates merely by free diffusion. The theoretical interpretation of these quantitative relationships will be treated in the discussion.

The absorption of actively transported substances such as sugars and amino acids usually involves concentration of these substances within the mucosal cell[11–15]. It therefore seemed desirable to determine the distribution of convallatoxin in the mucosal and serosal sections of the intestinal tissue after addition of the substrate to mucosal or serosal solutions. Table I shows the glycoside content of the two sections of the tissue after 45 min perfusion with a solution of 400 μM convallatoxin. When the substrate is added to the serosal solution, there is no significant difference in the convallatoxin content of the two sections of the tissue. In both compartments, it is approx. 10 % of the medium concentration. When the drug is dissolved in the mucosal solution, the mucosal section contains three times more glycoside than the serosal section. Control experiments demonstrated that this difference was not due to insufficient removal of adsorbed glycoside-containing perfusate. After perfusion with 400 μM convallatoxin for only 2.25 min and extraction of the gut in the usual manner, 1–14 nmoles convallatoxin per g wet weight were found in the mucosal section, whereas after 45 min perfusion, 116 nmoles/g wet weight were estimated.

In a further series of experiments, the time dependence of convallatoxin uptake by the tissue and passage into the opposite medium was gauged, when the glycoside was added only to the serosal solution. For this purpose, the intestine was perfused for 15, 30 or 45 min, and the convallatoxin content of mucosal solution, and mucosal and serosal sections of the tissue were determined at the end of the experiment (Fig. 3). After 15 min, a steady state is nearly attained, at which stage the drug

TABLE I

CONVALLATOXIN CONTENT IN PERFUSATE, INTESTINAL TISSUES, AND SEROSAL SOLUTION

Perfused rat small intestine *in vitro*. Convallatoxin concentration 400 μM in perfusate or serosal solution respectively. Composition of media see METHODS; temperature 37.5°, time 45 min. Values are expressed as mean ± S.E.M.; number of experiments in parentheses.

Convallatoxin supply	*Convallatoxin (nmoles/g wet weight or ml fluid)*				*Ratio mucosal section/serosal section*
	Perfusate	*Mucosal section*	*Serosal section*	*Serosal solution*	
Mucosal side	400	116.7 ± 13.0 (6)	40.0 ± 8.72 (6)	0.279 ± 0.065 (8)	3.26 ± 0.365 (6)
Serosal side	5.81 ± 0.53 (12)	52.6 ± 3.6 (10)	44.0 ± 9.0 (11)	400	0.960 ± 0.098* (8)

* $P < 0.0027$.

concentration in the mucosal section is 35 nmoles/g wet weight, and in the serosal section is 29 nmoles/g wet weight. In the next half hour, the concentrations increase to only 52 and 44 nmoles/g respectively. No difference between the concentrations in the two intestinal sections was ever statistically significant. Conversely, the concentration in the mucosal solution increases almost linearly from 1.3 μM after 15 min to 5.8 μM after 45 min.

At a certain stage in this work, the suspicion arose that the high penetration rate observed when 400 μM convallatoxin was introduced on the serosal side of the tissue might be due to release of mucosal tissue containing accumulated glycoside into the perfusate. For this reason, the protein content in both solutions and in mucosal and serosal sections of the gut tissue was determined. All measurements dis-

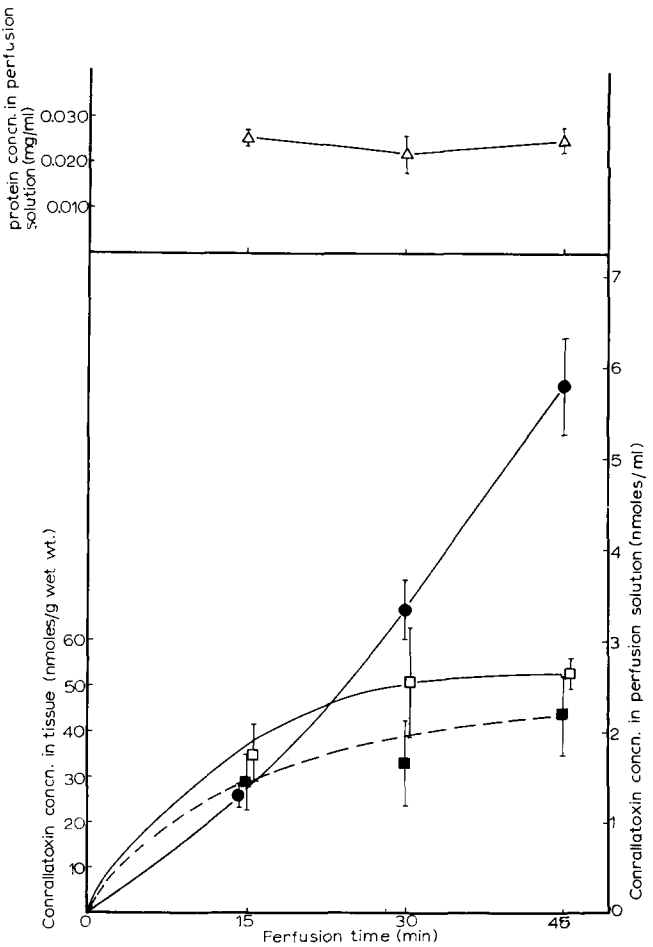

Fig. 3. Time course of convallatoxin penetration into the intestinal tissues and the perfusate (below) and of the protein extrusion into the perfusate (above). Glycoside introduced into serosal medium. perfused small intestine of the rat *in vitro*; composition of media see METHODS; temperature 37.5°. Abscissa: perfusion time; ordinate: convallatoxin concentration (below) and protein concentration (above). □—□, convallatoxin concentration in mucosal section of tissues; ■—■, convallatoxin concentration in serosal section of tissues; ●—●, convallatoxin concentration in the perfusate; △—△, protein concentration in the perfusate. Each point represents the mean from 4–13 experiments ± S.E.M.

pelled any possibility that desquamation might be responsible for the high penetration rate. In the time-dependence experiments, the protein content of the mucosal solution remained practically constant after 15 min, although the glycoside concentration increased continually (Fig. 3). Furthermore, the protein content of the media per ml was low in comparison with the protein content of the tissue per g wet weight. When the relationship between penetration of convallatoxin and the amount of protein simultaneously released into the medium is determined, it is found (Table II) that the quotient (expressed in nmoles convallatoxin per mg protein) is 300 times greater in the solution than in the adjacent tissue section when serosa–mucosa movement is estimated, and 5 times greater when mucosa–serosa transport is studied.

TABLE II

RELATIONSHIP BETWEEN CONVALLATOXIN AND PROTEIN CONTENT IN PERFUSATE, INTESTINAL TISSUES, AND SEROSAL SOLUTION

Perfused rat small intestine *in vitro*, convallatoxin concentration 400 μM in perfusate and serosal medium respectively. Experimental conditions see Table I. Values are expressed as mean $\pm$ S.E.M.; number of experiments in parentheses.

Convallatoxin supply	*nmoles convallatoxin per mg protein in*			
	Perfusate	*Mucosal section*	*Serosal section*	*Serosal solution*
Mucosal side		1.71 $\pm$ 0.39 (6)	0.307 $\pm$ 0.091 (5)	1.56 $\pm$ 0.48 (5)
Serosal side	254 $\pm$ 43 (11)	0.843 $\pm$ 0.036 (10)	0.387 $\pm$ 0.088 (11)	

DISCUSSION

Cortisol

The absorption kinetics of cortisol can be described without any discrepancy in terms of free diffusion. Its absorption rate was found by SCHEDL AND CLIFTON[16] to be essentially independent of its concentration in the lumen in experiments with perfused rat loops *in vivo*. Likewise, in our previous experiments with perfused rat intestinal loops *in vitro*, its absorption rate was found to be independent of the medium concentration of the steroid under all experimental conditions[3] and to be independent of Na^+ (ref. 4). Since cortisol is able to inhibit the absorption of convallatoxin[3], it cannot be entirely excluded that, as in the case of pyrimidines and purines[17,18], a transport process might be involved in cortisol absorption besides free diffusion. However, in view of the results mentioned above, a transport component may be considered negligible at the concentration levels employed in this study. It therefore appeared that cortisol was a suitable substance to test the penetration behaviour of free diffusing substances. The penetration rate is found to be independent of the steroid concentration when introduced either in the serosal or the mucosal solution. The penetration rate in the physiological direction (lumen $\rightarrow$ blood) is found to be three-fold greater than in the opposite direction. This might be attributed to the fact that the absorbed substances and fluid, which may be preferentially transported away by the lymphatic system[19], thus avoid the diffusion barrier afforded *in vitro* by the muscular layers and the serosa. So corticosteroids penetrating from the serosal solution are obliged to pass through additional tissue layers and also to diffuse against the flow of fluid preferentially absorbed from the mucosal solution. In sum-

mary, therefore, it appears that the penetration characteristics of a freely diffusing substrate, as studied by the method of FISHER AND PARSONS, agree with expectations, and so the preparation is deemed to be suitable for the study of the behaviour of transported substrates.

Convallatoxin

The penetration behaviour of convallatoxin is fundamentally different from that of cortisol. The penetration rate decreases with increasing substrate concentration whether the glycoside is introduced into the mucosal or the serosal solution. In the former case, this decline is particularly notable, an increase in substrate concentration from 5 to 400 μM inducing a decrease in relative penetration from 3.80 % to 0.175 %. The penetration of convallatoxin in the reverse direction shows a similar, but less pronounced concentration dependence. Since the penetration rate falls off less rapidly with increasing substrate concentration, there occurs the surprising phenomenon that when 400 μM convallatoxin is supplied, the transport is ten-fold faster in the unphysiological direction. However, this apparently paradoxical behaviour in fact provides a strong argument in favour of mediated transport when the theoretical relationships are considered.

According to WILBRANDT AND ROSENBERG[5], the velocity of a carrier-mediated transport may be described by the following equation:

$$v = v_{max} K_m \frac{(S_1 - S_2)}{(S_1 + K_m)(S_2 + K_m)}$$

in which v signifies the transport velocity of the substrate, v_{max} its maximal transport velocity, K_m the dissociation constant of the carrier–substrate complex, and S_1 and S_2 the substrate concentrations on the two sides of the membrane containing the transport mechanism.

Since sugars and amino acids are accumulated within the intestinal mucosal cells, the corresponding transport mechanisms must be localised in the luminal membrane of the epithelial cell[11–15]. The concentration of convallatoxin was also found to be three times higher in the mucosal than in the serosal section of the tissue after introduction into the mucosal solution. No significant differences between the glycoside content of the two halves of the tissue were demonstrated after serosal supply. (The observation that no equilibration of concentration occurs when the substrate is added to the mucosal medium—in contrast to the results after serosal supply—may be ascribed to removal of regularly absorbed glycoside through the lymphatic and blood vessels.) Therefore it seems justified to assume that the transport mechanism for cardiotonic steroids is also located in the luminal membrane of the mucosal cell. So, in the equation cited above, S_1 and S_2 will represent the convallatoxin concentrations in the perfusate and in the mucosal tissue, independent of the site of administration.

From the two series of experiments in which 400 μM convallatoxin was introduced into the luminal and the serosal solutions respectively, and the glycoside concentrations in the opposite solutions and the mucosal section were estimated, two independent equations can be derived. Both contain v_{max} and K_m as the sole unknown parameters, if some simplifications are adopted: The glycoside concentration in the mucosal section measured after 45 min perfusion time and the glycoside penetration

into the opposite solution have to be assumed to be constant during the whole period, since the cited equation is strictly valid only for steady-state conditions. Furthermore, the glycoside concentration in the wet mucosal section has to be regarded as representing the intracellular concentration of the mucosal cells. Making these assumptions, and solving the two resulting equations for K_m and v_{max}, K_m is calculated as 8.2 μmoles/l and v_{max} as 16 nmoles/cm gut per h. Due to the approximations made and considering, furthermore, that the gut is a complex organ consisting of different tissue layers and diffusion barriers, these values can only be regarded as rough estimates.

Nevertheless, the orders of magnitude obtained provide the following information: When convallatoxin is introduced into the mucosal solution, the glycoside concentration on both sides of the membrane, namely 400 μM and 120 μM, is far higher than a dissociation constant of 8 μM, with the result that the transport mechanism is almost completely saturated on both sides and the net transport is extremely low. When convallatoxin is administered on the serosal side, the concentration on the intracellular side of the transport mechanism (namely 53 μM) is considerably higher than this dissociation constant, whereas the concentration on the trans side (in the perfusate) is lower (6 μM after 45 min), with the result that the net transport rate should indeed be higher.

Strictly, the foregoing conclusions are only valid for a facilitated diffusion system. Since convallatoxin penetration is dependent on Na^+ (ref. 4), the possibility must be considered that the system may mediate uphill transport. The preceding arguments are not, however, disturbed by this possibility. CHRISTENSEN and coworkers[20,21] have shown that the intestinal absorption of sugars and amino acids is reversible. Furthermore, it is simple to demonstrate theoretically that the basic phenomenon, namely the reversal of the penetration rates on increasing the concentration of the glycoside, also occurs in the case of an active transport characterised by an increase in K_m on the inside of the membrane.

The results in the present paper therefore confirm the conclusion drawn from the previous work that cardiotonic steroids are absorbed in the intestine by a mediated transport system. It is not yet certain whether this system involves uphill transport nor is the nature of its physiological substrate yet clear.

In conclusion, two further aspects deserve mention. First, does this system also exist in other organs? Its presence in the liver is highly probable, since the concentration of convallatoxin in bile is much higher than in plasma[2]. Based on mathematical analyses of therapeutic and toxic effects of glycosides, WILBRANDT[22,23] concluded that the heart absorbs glycosides by mechanisms displaying saturation kinetics. The experiments described herein support this concept. Such considerations allow the notable accumulation of a relatively polar glycoside such as digoxin in the heart and some other tissues[24] to be interpreted in terms of a transport mechanism.

The second point concerns the general mechanism of the intestinal absorption of drugs. According to the theory of HOGBEN *et al.*[25], which has been verified on numerous occasions, drugs are absorbed by diffusion corresponding to their solubility in the lipoid cell membranes. The absorption characteristics of cardiotonic steroids demonstrates that this principal is not of general validity, and in certain cases may be replaced by mediated transport mechanisms.

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REFERENCES

1 F. LAUTERBACH AND G. VOGEL, *Arch. Pharmakol. Exptl. Pathol.*, 255 (1966) 37.
2 F. LAUTERBACH, *Arch. Pharmakol. Exptl. Pathol.*, 247 (1964) 391.
3 F. LAUTERBACH, *Arch. Pharmakol. Exptl. Pathol.*, 257 (1967) 432.
4 F. LAUTERBACH, *Arch. Pharmakol. Exptl. Pathol.*, 257 (1967) 458.
5 W. WILBRANDT AND T. ROSENBERG, *Pharmacol. Rev.*, 13 (1961) 109.
6 R. B. FISHER AND D. S. PARSONS, *J. Physiol. London*, 110 (1949) 36.
7 F. LAUTERBACH, *Biochim. Biophys. Acta*, 135 (1966) 256.
8 R. H. SILBER AND C. C. PORTER, *J. Biol. Chem.*, 210 (1954) 923.
9 F. NEUWALD, *J. Am. Pharm. Assoc.*, 39 (1950) 172.
10 F. DICKENS AND H. WEIL-MALHERBE, *Biochem. J.*, 35 (1941) 7.
11 R. K. CRANE AND P. MANDELSTAM, *Biochim. Biophys. Acta*, 45 (1960) 460.
12 D. B. MCDOUGAL Jr., K. D. LITTLE AND R. K. CRANE, *Biochim. Biophys. Acta*, 45 (1960) 483.
13 H. AKEDO AND H. N. CHRISTENSEN, *J. Biol. Chem.*, 237 (1962) 113.
14 J. W. L. ROBINSON, J.-Cl. JÉQUIER AND J.-P. FELBER, *Experientia*, 21 (1965) 12.
15 W. B. KINTER AND T. H. WILSON, *J. Cell Biol.*, 25 (1965) 19.
16 H. P. SCHEDL AND J. A. CLIFTON, *Gastroenterology*, 41 (1961) 491.
17 L. S. SCHANKER AND D. J. TOCCO, *J. Pharmacol. Exptl. Therap.*, 128 (1960) 115.
18 L. S. SCHANKER, J. J. JEFFREY AND D. J. TOCCO, *Biochem. Pharmacol.*, 12 (1963) 1047.
19 J. S. LEE, *Am. J. Physiol.*, 200 (1961) 979.
20 H. N. CHRISTENSEN AND S. J. GRAY, *Proc. Soc. Exptl. Biol. Med.*, 114 (1963) 215.
21 H. N. CHRISTENSEN, B. H. FELDMAN AND A. B. HASTINGS, *Am. J. Physiol.*, 205 (1963) 255.
22 W. WILBRANDT, K. BRAWAND AND P. N. WITT, *Arch. Exptl. Pathol. Pharmakol.*, 219 (1953) 397.
23 W. WILBRANDT, *Helv. Physiol. Acta*, 10 (1952) 372.
24 J. E. DOHERTY AND W. H. PERKINS, *Am. J. Cardiol.*, 17 (1966) 47.
25 C. A. M. HOGBEN, D. J. TOCCO, B. B. BRODIE AND L. S. SCHANKER, *J. Pharmacol. Exptl. Therap.*, 125 (1959) 275.