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The binding of ^{3}H-digitoxigenin by guinea-pig atrial tissue

K. KUSCHINSKY, H. LÜLLMANN AND P. A. VAN ZWIETEN

Department of Pharmacology, Christian-Albrechts-University, Kiel, West Germany

1. The uptake and release of ^{3}H-digitoxigenin by electrically driven, guinea-pig isolated atria (frequency 180/min) has been determined for two different medium concentrations of the genin. Both concentrations—1×10^{-7} and 5×10^{-7} g/ml.—caused a pronounced increase in contractile force.

2. The uptake of ^{3}H-digitoxigenin reached equilibrium after approximately 2 hr of incubation. At equilibrium, the tissue/medium radioactivity ratio reached a value of about 8 for both concentrations studied. After initial saturation of the atria with ^{3}H-digitoxigenin, approximately 60% of the accumulated genin was released on wash-out in genin-free Tyrode solution for 2 hr.

3. After presaturation of the atria with equimolar concentrations of either digitoxigenin or digitoxin (both non-radioactive) the uptake of ^{3}H-digitoxigenin occurred more slowly. After 2 hr of incubation approximately 60–70% of the tissue bound digitoxigenin or digitoxin had exchanged against ^{3}H-digitoxigenin from the medium. Presaturation of the atria with ouabain in equimolar concentration did not significantly affect the uptake of ^{3}H-digitoxigenin.

4. The release process of ^{3}H-digitoxigenin was not affected by the same concentration of non-radioactive digitoxigenin as used for the accumulation of the genin.

5. The kinetic properties of ^{3}H-digitoxigenin, determined by means of isotope techniques, are very similar to those of ^{3}H-digitoxin. The discrepancy between kinetic and pharmacological behaviour is, however, much larger for digitoxigenin than for digitoxin. Obviously, the presence or absence of the sugar moieties considerably influences the behaviour of the molecule towards the specific receptors, without particularly affecting the binding to unspecific, cellular structures.

Although in the molecule of digitoxigenin the sugar moieties are absent, the drug shows pronounced positive inotropic properties. While the positive inotropic action of digitoxigenin is not basically different from that of the usual cardiac glycosides, the absence of the sugar moieties causes considerable changes of the physicochemical properties which might explain the differences in resorption and duration of action between the genin and digitoxin (Rothlin & Bircher, 1954). In a number of papers we have described our investigations on the binding of radioactively labelled cardiac glycosides to isolated beating atrial tissue preparations.

The following drugs have been studied: ^{3}H-digoxin (Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967; Kuschinsky, Lüllmann, Schmitz & van Zwieten, 1967); ^{3}H-ouabain and ^{3}H-digitoxin (Kuschinsky, Lüllmann & van Zwieten, 1968a). A tritium labelled aglycone was put at our disposal, so we were able to extend these studies to ^{3}H-digitoxigenin and to compare its kinetic properties with those of the various cardiac glycosides. The results of these investigations suggest that the sugar moieties in the cardiac glycosides are not of particular importance for the kinetic behaviour in isolated heart muscle tissue.

Methods

The tritiation of digitoxigenin was carried out according to the Wilzbach procedure at the Institute for Nuclear Research at Karlsruhe (West Germany). The specific activity of the ^{3}H-digitoxigenin preparation that was finally obtained amounted to 0.6 mc/mg. Its purity was checked regularly by means of thin-layer chromatography, performed on silica gel + $CaSO_4$ (thickness 0.2 mm), a mixture of chloroform and methanol (95 : 5, v/v) being used as a solvent. The chromatograms were scanned by means of a Packard radiochromatogram scanning system. Only one radioactive peak could be detected. Its R_F value (0.40) proved the same as that of non-radioactive digitoxigenin.

All experiments were carried out on guinea-pig isolated atria. The details of the experimental method have been described in a preceding paper (Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967). Both the accumulation and the release of ^{3}H-digitoxigenin were determined as a function of the incubation period. The determination of total radioactivity in the atria was also carried out according to a procedure described in the aforementioned paper. By means of thin-layer chromatography it could be shown that ^{3}H-digitoxigenin is not metabolized in the given experimental conditions. Concomitantly, the total tissue radioactivity is a direct measure for the digitoxigenin concentration as such.

The exchange of tissue bound, non-radioactive digitoxigenin, digitoxin or ouabain against ^{3}H-digitoxigenin in the medium was always carried out at equilibrium. The experimental procedure of the exchange studies was virtually the same as described previously (Kuschinsky, Lüllmann, Schmitz & van Zwieten, 1967; Kuschinsky, Lüllmann & van Zwieten, 1968a, b). In all experiments the volume of the bath fluid was 250 ml., its temperature 30° C. The Tyrode solution contained Ca^{2+}, 1.2 m-equiv./l. Throughout the experiments the atria were stimulated by means of a Grass S 4 H stimulator with a frequency of 180/min. The positive inotropic effect of digitoxigenin was established in separate experiments by means of a strain gauge connected to a Helicoscriptor 86 HE-t recorder. In all calculations the extracellular space of atrial tissue was assumed to be 0.35 ml./g (Lüllmann & van Zwieten, 1967).

Results

The kinetic behaviour of ^{3}H-digitoxigenin was studied for two different concentrations of the genin, namely 1×10^{-7} and 5×10^{-7} g/ml. These concentrations are 2.7×10^{-7} and 1.3×10^{-6}M, respectively. The lowest concentration increased the

contractile force by $47 \pm 5.4\%$ of the original value (mean $\pm$ S.E.M., $n=17$, determined on several atria). The highest concentration increased the contraction amplitude by $153 \pm 19\%$ ($n=8$). After prolonged incubation (>30 min) in a Tyrode solution that contained digitoxigenin 5×10^{-7} g/ml. the atria sometimes developed contractures. Accordingly, in some cases the highest concentrations already reached the toxic range, whereas the lowest concentration showed only a moderate therapeutic action.

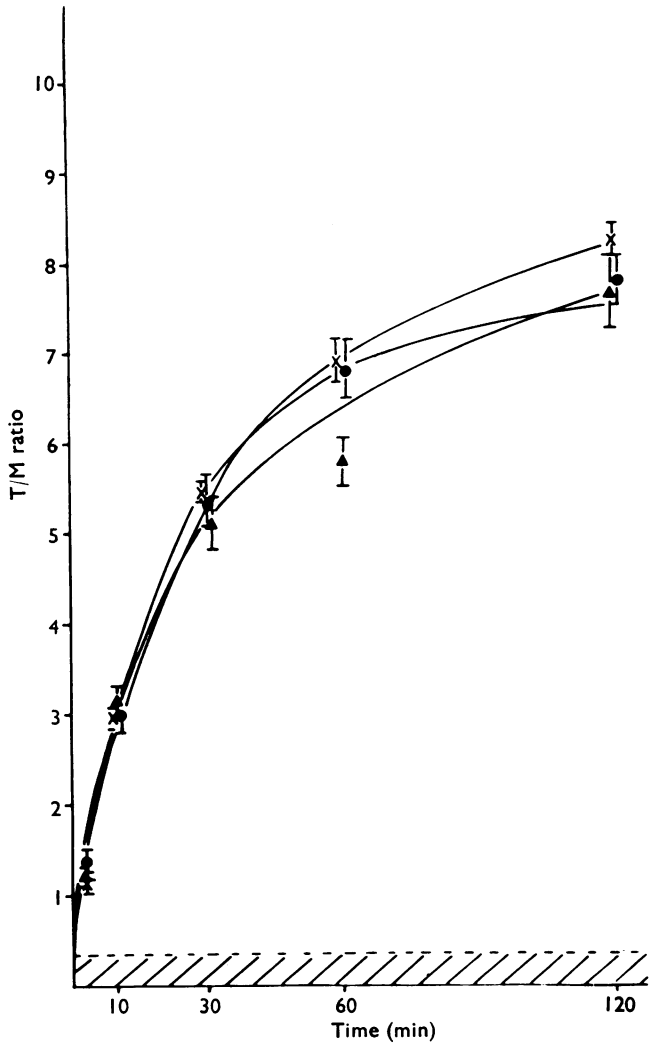

FIG. 1. Uptake of ^{3}H-digitoxigenin. Experiments with electrically driven, isolated atria (guinea-pig). Before the actual uptake process the atria were equilibrated in genin-free Tyrode solution for 30 or 150 min. Ordinate: Tissue/medium radioactivity ratio (T/M ratio). Abscissa: Incubation time in the solution containing ^{3}H-digitoxigenin. The hatched part of the figure represents the relative amount of radioactive material present in the extracellular space. Each point on the curves represents the mean value (±S.E.M.) of eight or ten atria. ▲——▲, Digitoxigenin 1×10^{-7} g/ml. Tyrode solution; equilibration for 30 min in genin-free solution. ●——●, Digitoxigenin 5×10^{-7} g/ml. Tyrode solution; equilibration for 30 min in genin-free solution. ×——×, Digitoxigenin 5×10^{-7} g/ml. Tyrode solution; equilibration for 2.5 hr in genin-free solution.

Uptake and release of ^{3}H-digitoxigenin

The tissue uptake of ^{3}H-digitoxigenin was measured for both concentrations up to 2 hr. The tissue/medium radioactivity (T/M ratio) was plotted as a function of the incubation time (Fig. 1). For both concentrations of medium the uptake reached equilibrium after approximately 2 hr. At equilibrium the T/M ratio was approximately 8. The amount of ^{3}H-digitoxigenin present in the extracellular space is also shown in Fig. 1. By means of the equation $Y = A\ (1 - e^{-kt})$ the rate constant k could be determined graphically. In this equation Y represents the uptake at time t, A the uptake at equilibrium. The rate constant (k) of the uptake process was calculated from the slope of the straight line, obtained by plotting t versus $\ln \frac{A}{A-Y}$. This rate constant amounted to 5.7×10^{-4} sec^{-1}. By means of the equation $t\frac{1}{2} = \frac{0.693}{k}$ the half-life ($t\frac{1}{2}$) of the uptake process was calculated as approximately 20 min. Neither the rate of the uptake process nor the T/M ratio at equilibrium were significantly different for the two digitoxigenin concentrations investigated.

Two of the uptake curves shown in Fig. 1 were obtained after initial equilibration of the isolated atria in genin-free Tyrode solution for 30 min. With regard to the exchange processes, it seemed of interest to establish whether a longer equilibration

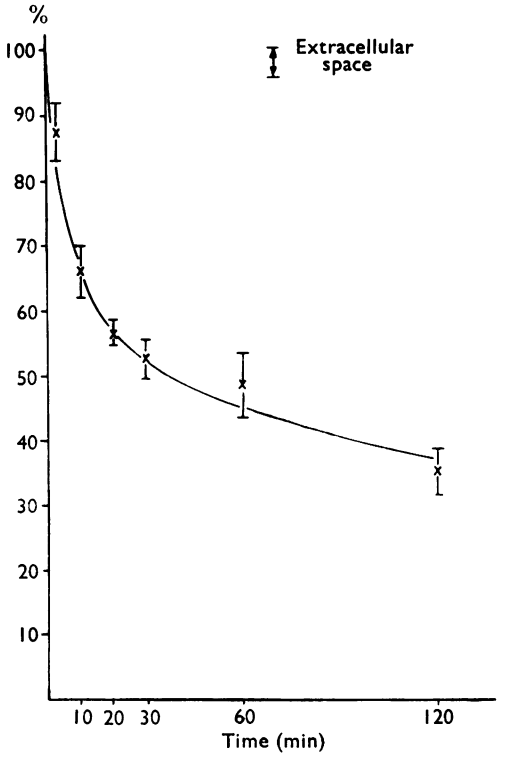

FIG. 2. Release of previously bound ^{3}H-digitoxigenin on incubation of the atria in genin-free Tyrode solution. After accumulation of ^{3}H-digitoxigenin until equilibrium was reached (medium concentration 1×10^{-7} g/ml.) the atria were transferred to glycoside-free Tyrode solution and incubated for various periods. The radioactivity of the organs was expressed as a percentage of the initial tissue radioactivity and subsequently plotted against the incubation time in the glycoside-free Tyrode solution. Each point on the curve represents the mean value (±S.E.M.) of at least ten atria. The relative amount of radioactive material which occupies the extracellular space has been represented by an arrow.

in Tyrode solution could influence the uptake process. Fig. 1 also shows the uptake of ^{3}H-digitoxigenin by atria that have been equilibrated in Tyrode solution during 2.5 hr before the actual uptake experiment, instead of 30 min as usual. Obviously, the prolongation of the equilibration period from 30 min to 2.5 hr did not influence the uptake process, because the curves are not significantly different.

In order to establish the rate by which isolated atria may release previously accumulated ^{3}H-digitoxigenin, the organs were incubated for 2 hr in a solution containing ^{3}H-genin until equilibrium had been reached and subsequently transferred to a genin-free Tyrode solution. The residual radioactivity (in % of the maximal value) was plotted as a function of the incubation period in the genin-free Tyrode solution (Fig. 2). The curve in Fig. 2 was obtained for a medium concentration of 1×10^{-7} g/ml. As shown in Fig. 4 ($\times$——$\times$) approximately the same curve was obtained for a bath concentration of 5×10^{-7} g/ml. Thus, within the range studied, the release of ^{3}H-digitoxigenin is also virtually independent of the concentration of the loading medium. The release process occurred rather slowly, because it took about 1 hr until half of the initially accumulated ^{3}H-digitoxigenin

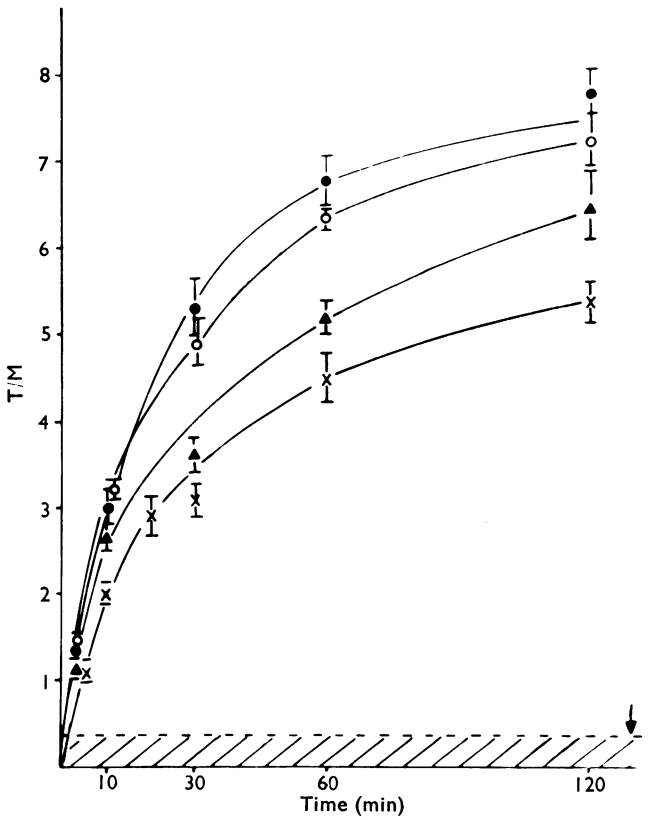

FIG. 3. Uptake of ^{3}H-digitoxigenin (5×10^{-7} g/ml.) after presaturation of the atria with equimolecular concentrations of non-radioactive digitoxigenin, digitoxin or ouabain. The uptake of ^{3}H-digitoxigenin by aglycone-free atria is also shown (same curve as in Fig. 1, ●——●). Details see text and Fig. 1. ●——●, Uptake of ^{3}H-digitoxigenin by genin-free atria. ▲——▲, Uptake of ^{3}H-digitoxigenin after presaturation of the organs with non-radioactive digitoxigenin. $\times$——$\times$. Uptake of ^{3}H-digitoxigenin after presaturation of the organs with non-radioactive digitoxin. ○——○, Uptake of ^{3}H-digitoxigenin after presaturation with non-radioactive ouabain.

had been washed out. After an incubation period of 2 hr in the genin-free, radioactive Tyrode solution approximately 40% of the accumulated ^{3}H-digitoxigenin remained bound by the atrial tissue.

Exchange of tissue-bound, non-radioactive digitoxigenin or cardiac glycosides against ^{3}H-digitoxigenin in the medium ("uptake-exchange")

The exchange process of tissue-bound, non-radioactive digitoxigenin against ^{3}H-digitoxigenin in the medium was studied at equilibrium for a bath concentration of digitoxigenin 5×10^{-7} g/ml. After an initial incubation of 2 hr in a medium containing non-radioactive digitoxigenin, the uptake process will have reached the equilibrium phase (see Fig. 1). The atria thus treated were transferred to a Tyrode solution containing ^{3}H-digitoxin 5×10^{-7} g/ml. and incubated for various periods. As shown in Fig. 3, the uptake of ^{3}H-digitoxigenin was significantly inhibited by the preceding saturation of the tissues with the non-radioactive aglycone. About 60–70% of the tissue-bound digitoxigenin had exchanged against ^{3}H-digitoxigenin from the medium after 2 hr. In separate experiments we studied the uptake of ^{3}H-digitoxigenin by organs that had been treated with non-radioactive digitoxin

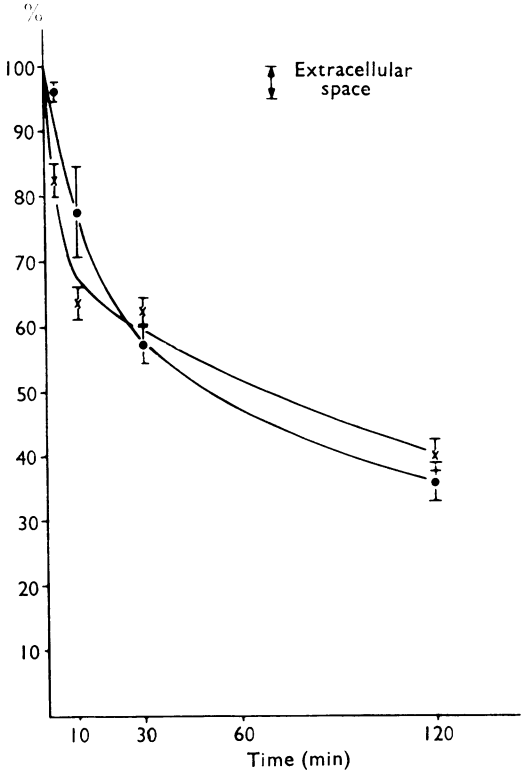

FIG. 4. Release of ^{3}H-digitoxigenin and exchange (cell→extracellular space). Experiments with guinea-pig isolated atria. For details, see text and Figs. 1 and 2. Digitoxigenin content of the medium, 5×10^{-7} g/ml. Ordinate, tissue radioactivity in % of the initial value. Abscissa, incubation time of the ^{3}H-digitoxigenin-loaded atria in either genin-free or genin-containing Tyrode solution (release or exchange, respectively). Each point on the curves represents the means value (±S.E.M.) of eight or ten atria. ×——×, Release; ●——●, exchange.

until the accumulation of this cardiac glycoside had reached the equilibrium phase. The digitoxin content of the medium was 1×10^{-6} g/ml.—a concentration equimolecular with that of the ^{3}H-digitoxigenin solution (5×10^{-7} g/ml.). The uptake of digitoxin achieves the equilibrium stage after 2 hr of incubation (Kuschinsky, Lüllmann & van Zwieten, 1968a). As demonstrated in Fig. 3, the presaturation of the isolated atria with digitoxin also provoked a considerable impairment of the ^{3}H-digitoxigenin accumulation in comparison with digitoxin-free organs. This inhibition proved to be of approximately the same degree as that provoked by presaturation of the heart muscle tissue with digitoxigenin. As described earlier the mere fact of a longer incubation period cannot as such be the cause of the impaired ^{3}H-digitoxigenin uptake. A pronounced prolongation of the equilibration period (from 30 min to 2.5 hr) did not influence the ^{3}H-digitoxigenin uptake process.

We also investigated whether presaturation of isolated atria with non-radioactive ouabain might influence the accumulation of ^{3}H-digitoxigenin. For this purpose the organs were incubated for 30 min in a Tyrode solution containing ouabain 7.8×10^{-7} g/ml. Again, this ouabain concentration was equimolecular with that of ^{3}H-digitoxigenin (5×10^{-7} g/ml.) used in the uptake experiments. In contrast to the digitoxin experiments, presaturation of the tissue with ouabain did not impair the ^{3}H-digitoxigenin uptake process (Fig. 3).

Exchange of tissue-bound ^{3}H-digitoxigenin against non-radioactive digitoxigenin in the bath fluid ("release-exchange")

This exchange process was determined for one medium concentration of digitoxigenin (5×10^{-7} g/ml.). After pre-incubation of the organs with ^{3}H-digitoxigenin for 2 hr the release of the initially accumulated, radioactively labelled genin was determined over a period of 2 hr. The experiments were again carried out at equilibrium. Accordingly, the Tyrode solution to which the "radioactive" atria were transferred contained the same concentration (5×10^{-7} g/ml.) of non-radioactive digitoxigenin. As shown in Fig. 4, the presence of non-radioactive digitoxigenin in the bath fluid did not significantly influence the release of ^{3}H-digitoxigenin by isolated atria.

Discussion

The present paper deals with the problem of how far the kinetic behaviour of an aglycone is different from that of the corresponding cardiac glycoside. The conclusions reached are based on the results of isotope studies compared with the pharmacological properties of the drug.

Comparison of the kinetic properties of digitoxigenin and digitoxin

The accumulation of ^{3}H-digitoxigenin by isolated atria showed approximately the same picture as that obtained for the uptake of ^{3}H-digitoxin (Kuschinsky, Lüllmann & van Zwieten, 1968a). Although the maximum of the uptake process was achieved somewhat earlier for ^{3}H-digitoxin, the uptake curve obtained for ^{3}H-digitoxigenin had essentially the same shape as that determined for the ^{3}H-labelled cardiac glycoside. The rate constant (k) of the ^{3}H-digitoxigenin accumulation (5.7×10^{-4} sec^{-1}) proved lower than that of the ^{3}H-digitoxin uptake (14×10^{-4} sec^{-1}), established in preceding studies (Kuschinsky, Lüllmann & van Zwieten, 1968a). The

T/M ratio in the equilibrium phase, however, was virtually the same for both drugs (9–10 for digitoxin, 8 for the genin). Consequently, a considerable cellular accumulation must have taken place for ^{3}H-digitoxigenin, the contribution of the extracellular space to the total tissue radioactivity being of minor importance (see Fig. 1). A striking similarity between the kinetic properties of digitoxin and its aglycone is also suggested by our observation that presaturation of the atria with either digitoxigenin or digitoxin (both non-radioactive) impaired the uptake of ^{3}H-digitoxigenin by approximately the same degree. Concomitantly, the same type of exchange processes seem to occur in both cases: the ^{3}H-digitoxigenin in the medium exchanges against either non-radioactive digitoxigenin or digitoxin, initially bound to cellular structures.

The exchange of tissue-bound cardiac glycosides against radioactively labelled glycosides in the medium and vice versa has been described in detail (Kuschinsky, Lüllmann, Schmitz & van Zwieten, 1967; Kuschinsky, Lüllmann & van Zwieten, 1968a, b). The results obtained for the exchange experiments with digitoxigenin are in satisfactory agreement with those already described for ouabain, digoxin or digitoxin (see above). The dissociation of the aglycone or glycoside molecules from the cellular structures to which they are attached seems to be the rate-limiting step in the exchange process. The presence of non-radioactive digitoxigenin in the medium did not influence the release of ^{3}H-digitoxigenin from the atria. If indeed the dissociation is the rate-limiting step, it cannot be influenced by the presence of non-radioactive digitoxigenin molecules in the bath fluid. Also in this respect the genin seems to behave like digitoxin.

In our preceding paper on ^{3}H-digitoxin (Kuschinsky, Lüllmann & van Zwieten, 1968a) it has been explained that the greater part of the accumulated cardiac glycoside is probably not of any importance to the positive inotropic effect of the drug. ^{3}H-ouabain, on the other hand seems to combine chiefly with more specific receptors that are pertinent to the development of the positive inotropic effect. The similarity between the uptake processes of digitoxin and its aglycone and especially the high T/M ratio at equilibrium suggest that probably digitoxigenin also accumulates in a mainly unspecific manner: the genin molecules equally combine with cellular structures which are not of any importance to the development of a positive inotropic action. Recent fractionation experiments by Gerber, Fricke, Klaus & Wollert (1968) have demonstrated that in myocardial tissue ^{3}H-digitoxin is bound by the nuclei and membranes, but also recovered from the cellular fraction that contains the microsomes and T-tubes. According to these authors, the accumulation by the microsomes and T-tubes may be correlated to the positive inotropic action of the drug, whereas the combination of ^{3}H-digitoxin with the other structures seems to be devoid of pharmacological significance. It might well be that the partition of ^{3}H-digitoxigenin between the various cellular fractions follows the same pattern as that observed for ^{3}H-digitoxin.

To recapitulate, the overall kinetic behaviour of digitoxin and digitoxigenin on isolated heart muscle preparations seems to be approximately the same.

Comparison of the kinetic properties of digitoxigenin and ouabain

As described previously, after exposure of isolated atria to ^{3}H-ouabain, the drug is found chiefly in the extracellular space but to a minor degree attached to cellular

structures (Kuschinsky, Lüllmann & van Zwieten, 1968a). The small amount of ^{3}H-ouabain bound by the cell (probably located at the membrane or in the T-tube system) is possibly essential for the development of the positive inotropic action. The uptake of ^{3}H-digitoxigenin was virtually the same if the atria had been pre-saturated with an equimolecular concentration of non-radioactive ouabain. Obviously, the occupation of certain structures in or on the membrane by ouabain does not impair the penetration of the genin into the cell. If indeed ouabain combines with receptors which are probably located in or on the membrane, this observation also suggests that the greater part of the accumulated digitoxigenin is stored in cellular compartments that have nothing to do with the pharmacological effect. In this respect there is a sharp contrast between the behaviour of ^{3}H-digitoxin and its genin on the one hand and that of ^{3}H-ouabain on the other hand. These differences might be explained by the differences in polarity of the three drugs. Ouabain is far more polar than the lipid soluble compounds digitoxin and digitoxigenin (Waldi, 1962). There is little difference in polarity between digitoxin and its aglycone. Moreover, the genin is bound to serum proteins to approximately the same degree as digitoxin (~90% bound ; Kuschinsky, unpublished experiments).

Comparison of radiochemically obtained release curves and the wash-out of the pharmacological effect of digitoxigenin

Equimolecular concentrations of ouabain, digitoxin and digitoxigenin cause approximately the same positive inotropic effect ; the latter develops with the same time course. The rate at which a given increase in contractile force is dissipated on wash-out, however, considerably differs for the three drugs. It took about 25 min before the positive inotropic effect of digitoxin was reduced to 10% of its initial value on wash-out. For a similar reduction of a comparable positive inotropic effect produced by ouabain, wash-out for approximately 10 min is necessary. On the other hand, an almost complete disappearance of an increase in contractile force, provoked by digitoxigenin is achieved in 1–2 min (Lüllmann, Weber & van Zwieten, to be published). The picture that emerges from the radiochemical investigations is, however, entirely different. Both for ^{3}H-digitoxin and ^{3}H-digitoxigenin, about 40% of the initial radioactivity was left in the organ after a wash-out period of 2 hr, whereas for ^{3}H-ouabain the corresponding figure was approximately 25%. Obviously there is no correlation whatsoever between the rate at which the cardial glycosides and the aglycone are released and the rate of disappearance of the pharmacological effect. This lack of correlation indicates that in isolated atria the *total* tissue content of cardiac glycosides and digitoxigenin is probably not of particular importance for the pharmacological effect.

Conclusions

The kinetic investigations on isolated heart muscle preparations as described in the present and in preceding papers do not necessarily enable relevant conclusions to be drawn concerning the *in vivo* effect of cardiac glycosides and aglycones. The parameters determined by means of the isotope technique cannot be related to the *in vivo* actions of such drugs and their time course. *In vivo* there are several extracardial factors which may contribute to the overall kinetic behaviour of the cardioactive drugs. For example, factors such as protein binding, deposition in various tissue, metabolism and elimination through the kidneys and the liver

strongly influence the overall effects. Most likely the rate of renal elimination is specially important in determining the serum concentration of the drugs, although the differences in serum protein binding prevent the establishment of a direct relationship between serum level and pharmacological effect. Our kinetic studies on isolated atria suggest that the well known clinical differences of action between the various cardiac glycosides and aglycones are probably not caused by different kinetic behaviour in the heart muscle tissue as such. It seems much more likely that extracardial factors are responsible for the differences in clinical action.

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