Br. J. Pharmac. (1969), **36**, 276–285.

The distribution of ^{3}H-labelled cardenolides between isolated guinea-pig atrial tissue and circulating, oxygenated whole blood

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1. An experimental method was developed that allowed the incubation of isolated organs in circulating whole blood. The circulating blood was oxygenated with a specially designed disc-oxygenator and drawn through the system by means of a roller pump.

2. The method proved suitable for guinea-pig isolated atria, rabbit duodenum and to a lesser extent for chronically denervated rat diaphragm. Isolated atria could be kept for several hours. Various parameters of the circulating blood (haemolysis, pH, O_2 saturation, concentration of electrolytes) remained satisfactory for at least 5 hr. The method proved convenient for pharmacological and kinetic studies on isolated organs, suspended in whole blood of the corresponding species. The organs showed normal spontaneous mechanical activity and also responded to electrical stimuli and various drugs.

3. The uptake of ^{3}H-labelled ouabain, digoxin, digitoxin and digitoxigenin was studied in guinea-pig isolated atria, suspended in circulating blood. The uptake reached equilibrium after 60–90 min. With respect to the total serum radioactivity the "apparent" tissue/medium (T/M) ratios obtained for the four drugs were within the range 0·4–1·1. If, however, the amount of free, non protein bound drug was taken as a base for the calculations, the following "true" T/M ratios were obtained: ^{3}H-ouabain 0·45; ^{3}H-digoxin 1·6; digitoxin 8·8; digitoxigenin 8·4. These values are virtually the same as those obtained with atria, suspended in an aqueous medium. Obviously, the uptake of ^{3}H-cardenolides from whole blood is determined by the amount of free non-protein-bound drug.

4. ^{3}H-digitoxin and ^{3}H-digitoxigenin were taken up by guinea-pig erythrocytes to a small extent. No measurable amounts of ^{3}H-ouabain and ^{3}H-digoxin were taken up by erythrocytes.

The use of arterial whole blood for the incubation of isolated organs is desirable because the conditions in such experiments approach the physiological norm. Excessive foam formation, haemolysis and poor oxygenation have so far prevented the use of artificially oxygenated blood in experiments with isolated organs. Incubation in blood oxygenated in intact animals and subsequently led into the organ bath then reinfused into the animal circulation has already been described by Vane (1964).

By means of a specially designed disc-oxygenator, based on the principle of the heart-lung machine in cardiac surgery, however, we have been able to develop an experimental procedure, suitable for the incubation of isolated atria and other organs for periods exceeding several hours. The method described in the present paper proved suitable for kinetic studies involving the distribution of radioactively labelled drugs between isolated organs and various elements of the blood, used for incubation. In connection with studies on the kinetic behaviour of radioactively labelled cardiac glycosides in isolated atria suspended in Tyrode solution (Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967; Kuschinsky, Lüllmann, Schmitz & van Zwieten, 1967; Kuschinsky, Lüllmann & van Zwieten, 1968 a,b,c), it seemed of interest to extend these studies to experiments on guinea-pig atria, incubated in circulating, oxygenated whole blood. In such experiments not only is the nutrition of the tissue better, but such factors as binding to serum proteins influence uptake in a way more closely resembling the conditions *in vivo*.

Methods

Experimental details of the disc-oxygenator are shown in Fig. 1. The disc-oxygenator, consisting of thirty-two parallel circular discs (diameter 3·0 cm) perpendicular to a longitudinal axis (length 18 cm) was made of Perspex. The discs rotated 18 times/min. The whole system was treated with a solution of silicone oil in ether and subsequently washed out with Tyrode solution.

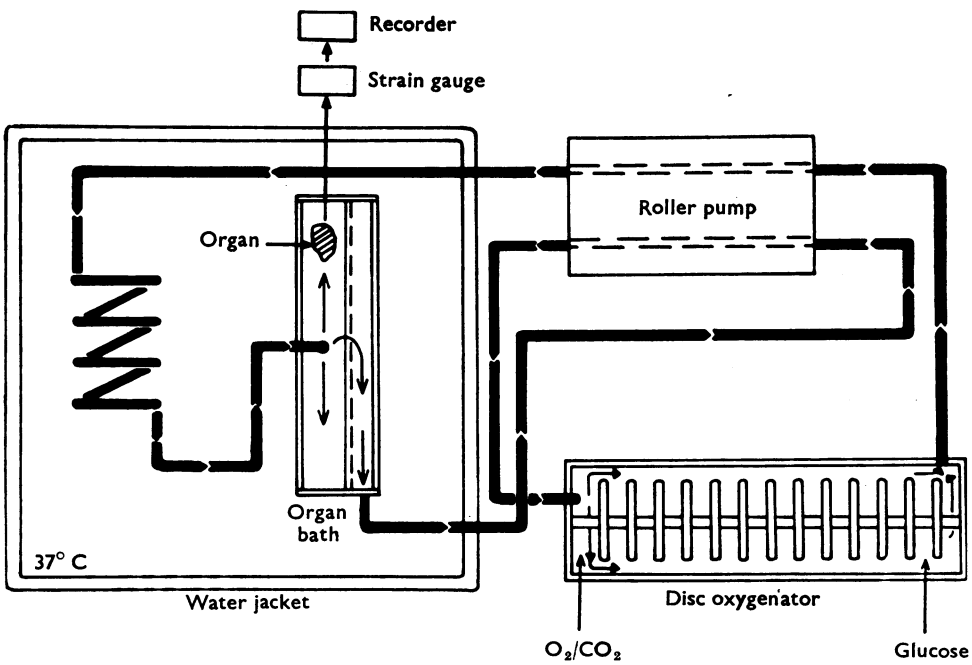

FIG. 1. Diagram of the circulation system, used for the suspension of isolated organs in oxygenated whole blood. Carbogen (95% O_2+5% CO_2) was led into the disc-oxygenator. Glucose solution was infused throughout the experiment.

Blood was drawn through the system by means of a roller pump (rate 40 ml./min). The blood was transported in such a manner that minimal compression occurred. The total volume of circulating blood amounted to 100 ml. A mixture of 95% oxygen and carbon dioxide was forced through the oxygenator. Simultaneously, 3·8 ml./hr of an isotonic glucose solution (5·2%) was added continuously to the circulation. The bath used for the incubation of the isolated atria also consisted of perspex. Its volume was 30 ml. About fifteen guinea-pig atria could be incubated simultaneously. The bath was divided into two compartments and designed in such manner that the formation of gas bubbles in the effluent medium could be avoided. Platinum electrodes were attached to the inside walls of the organ bath in order to allow electrical stimulation of the organs. The atria were stimulated with rectangular pulses obtained from a Grass S 4 H device (frequency 180/min. duration 4 msec, voltage 4–8 V). The temperature of the bath and that of the circulating blood was kept at 30°.

The mechanical activity of one single atrium could be determined by means of a strain gauge and corresponding amplification device. The contractions could thus be recorded continuously on a Helco-Scriptor recording device, type HE-86-t. Drugs were injected into the circulation until the appropriate final concentration was reached.

Guinea-pigs of either sex (250–350 g) were killed and bled from the carotids. About 40 i.u. of heparin (Heparin Novo (R)) per ml. of blood were added. The heparinized blood was filtered through cotton tissue. Guinea-pig isolated atria were prepared according to the procedure, described by Hoditz & Lüllmann (1964). The determination of total radioactivity in atrial tissue was carried out according to the procedure published previously (Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967). In order to raise the efficiency of counting, the samples were bleached by addition of 0·1 ml 30% aqueous H_2O_2. Radioactivity in serum was determined using a method already described (Lahrtz & van Zwieten, 1968).

The total radioactivity of the red cells was established by means of a specially developed method.

A sample (2 ml.) of whole blood was centrifuged (2,500 rev/min, for 5 min) and the serum removed by suction. The residual erythrocytes were suspended in 10 ml. of a buffer solution, composed as follows: 7·2 g NaCl, 1·0 g $NaHCO_3$, 0·18 g KCl, 0·14 g $CaCl_2$ per l. H_2O (pH 7·8). This buffer is isotonic with guinea-pig blood and also has the same pH. After shaking, the suspension was centrifuged as before, the supernatant removed by suction, 10 ml. of buffer solution added and the red cells again suspended. The mixture was centrifuged once more and the supernatant removed again until 0·5 ml. of the red cell suspension was left in the test tube. Distilled water (9·5 ml.) was added to the residual erythrocytes to produce complete haemolysis. A sample of 0·05 ml. was taken from the resulting haemolysate, bleached and added to a counting vial that contained a mixture of 2 ml. of absolute ethanol and 13 ml. of a scintillation phosphor (4 g PPO + 0·1 g POPOP, dissolved in 1000 ml. of anhydrous toluene). Thixotropic gel powder (0·8 g) (Packard Instrument Inc., Frankfurt) was added to the mixture. The samples were counted in a Packard liquid scintillation spectrometer. The individual quenching of the samples was determined by means of an external Ra-standard. The counting efficiency proved to be approximately 20%, and the counting error less than 5%.

Radioactive drugs

^{3}H-ouabain and ^{3}H-digitoxin were obtained from New England Nuclear, Inc., Chicago, ^{3}H-digoxin from Burroughs Wellcome Inc., Tuckahoe, New York. ^{3}H-digitoxigenin was prepared at the Institute for Nuclear Research at Karlsruhe and purified by the isotope department of Merck AG., Darmstadt.

Chromatography

The purity of the radioactive drugs was checked by means of thin layer chromatography, and the dried chromatograms were scanned in a Packard Radiochromatogram scanning system. The experimental details have been described in previous papers. All drugs used proved chromatographically pure. The following R_F values were obtained: ^{3}H-ouabain 0·7 ; ^{3}H-digoxin 0·6 ; ^{3}H-digitoxin 0·75 ; ^{3}H-digitoxigenin 0·5. These values are in reasonable agreement with those found in previous studies in which the same chromatographic systems were used. It could be demonstrated, that, even after a period of 4 hr, no measurable metabolic degradation of three of the cardiac glycosides had taken place in circulating guinea-pig blood. The serum contained only the original drugs and no metabolites. ^{3}H-digitoxigenin, however, proved to be metabolized in guinea-pig blood. On the thin layer chromatogram two radioactive peaks appeared with R_F values of 0·2 and 0·5. The peak with $R_F = 0·2$ must be attributed to a metabolite, the other peak represents unchanged ^{3}H-digitoxigenin. The metabolite became visible on the chromatogram after 10 min of incubation and increased with time. After 4 hr of incubation about half the radioactivity was due to the metabolite. No attempt was made to elucidate the structure of this compound.

Results

As will be discussed below, the use of oxygenated blood in the circulation device proved suitable for the maintenance of isolated organs in physiological conditions. Various parameters of the blood were determined during the experiments and the behaviour of the isolated organs was also observed. When the method had been proved reliable, distribution studies with tritium labelled cardiac glycosides were carried out in a system consisting of guinea-pig isolated atria, suspended in oxygenated whole blood of the same species.

Changes in the blood during circulation

The changes in various parameters of the oxygenated blood during circulation are listed in Table 1. They demonstrate that during circulation over periods up to 4 hr

TABLE 1. *Haemoglobin content, pH, O_2-saturation and ion concentrations in whole blood or serum during increasing periods of circulation*

Time (min)	Haemoglobin in serum (mg %) ±S.E.M.	pH whole blood	O_2 saturation whole blood %	(K^+) serum m-equiv./l. ±S.E.M.	(Na^+) serum m-equiv./l. ±S.E.M.	(Ca^{2+}) serum m-equiv./l. ±S.E.M.
0	29±3	7·8	97	8·2±0·4	111±9	2·1±0·11
30	29±4	7·8	97	7·8±0·2	111±13	2·1±0·13
120	33±3·5	7·8	97	7·6±0·5	98±11	Not detd.
240	49±6	7·8	97	7·6±0·8	98±7	2·25±0·09

there were no important changes from the normal physiological values. Slight changes in ion-concentrations were caused by the continuous infusion of isotonic glucose solution (3·80 ml./hr).

Behaviour of the incubated organs

Three different types of isolated muscle preparation were investigated in order to test the experimental procedure: atrial tissue (guinea-pig atria), smooth muscle (rabbit isolated duodenum) and striated muscle (chronically denervated rat diaphragm).

Guinea-pig isolated atria showed entirely satisfactory spontaneous mechanical activity. Moreover, the response to electrical stimuli and also that to various cardioactive drugs proved approximately the same as that in atria, suspended in Tyrode solution (see Fig. 2). Figure 3 shows the dose response curves for acetylcholine, isoproterenol and Ca^{2+} -ions. The dose response curves for each drug in atria suspended in circulating whole blood were compared with those for atria suspended in Tyrode solution. Because of the cholinesterase in the blood, the effect of acetylcholine disappeared much more rapidly in blood than it did in Tyrode solution. Obviously, there are no important differences between the two curves for acetylcholine shown in Fig. 3. The response to ouabain was also the same in both media (ED50 = $5{\cdot}5 \times 10^{-7}$M in both cases). In order to obtain a positive inotropic response to adrenaline in atria, suspended in circulating blood, rather high final concentrations (10^{-4}–10^{-3}M) were necessary. As described later, atria suspended in whole blood were also less sensitive to cardiac glycosides (with the exception of ouabain). Higher

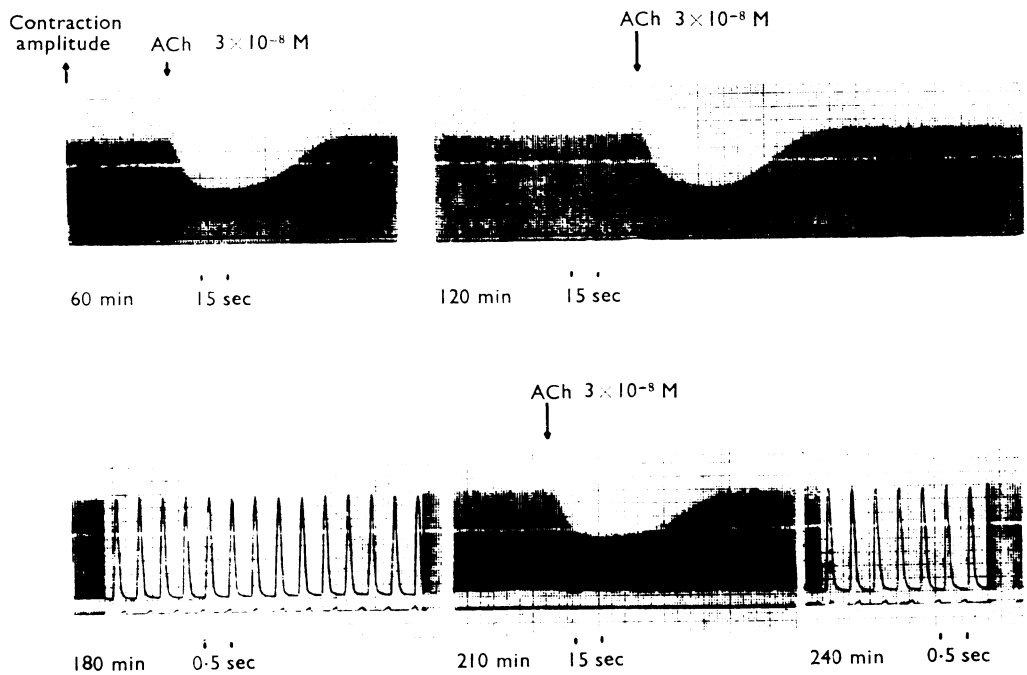

FIG. 2. Mechanical activity of a guinea-pig isolated atrium, suspended in oxygenated whole blood. The response to acetylcholine is also shown.

concentrations of these drugs were required for a positive inotropic action in blood-bathed organs than for organs incubated in Tyrode solution.

Pieces of isolated rabbit duodenum could be kept in circulating rabbit blood for at least 7 hr, the spontaneous peristaltic movements remaining entirely "normal" over this period. Moreover, acetylcholine increased the tone and the spontaneous activity of the organ, while adrenaline inhibited it.

Chronically denervated rat diaphragm could be maintained in circulating rat blood for approximately 90 min. The organ showed normal responses to acetylcholine. However, the oxygenation of this organ seemed less satisfactory than that of atria or isolated smooth muscle preparations.

The results obtained suggest that the method is suitable for pharmacological studies on various isolated organs.

Uptake of ^{3}H-labelled cardenolides by isolated atria and erythrocytes

The uptake was studied for subthreshold concentrations of ^{3}H-ouabain ($9{\cdot}9 \times 10^{-8}$M), ^{3}H-digitoxin ($6{\cdot}5 \times 10^{-7}$M) and ^{3}H-digitoxigenin ($2{\cdot}2 \times 10^{-6}$M). No positive inotropic effect was induced on addition of the radioactively labelled drug to the circulating blood, although in Tyrode solution these concentrations would be pharmacologically active. The absolute amount of radioactive material was rather high (about 50 μc/100 ml. blood). This concentration of radioactive material enabled us to trace even minute amounts of the drugs in the various elements of the system. In addition, the uptake of ^{3}H-digoxin was studied for two different bath concentrations, that is $3{\cdot}2 \times 10^{-7}$ and $3{\cdot}2 \times 10^{-6}$M. The lower concentration did not induce any positive inotropic action, whereas the higher toxic concentration gave rise to arrhythmias and contracture.

The uptake of the four ^{3}H-labelled drugs by isolated atria and by erythrocytes is shown in Fig. 4a and b. In Fig. 4a, the ratio tissue/serum radioactivity in atria and red cells, is plotted as a function of the incubation time. The uptake processes reached equilibrium after 60–90 min. Apparently, there are no important differences

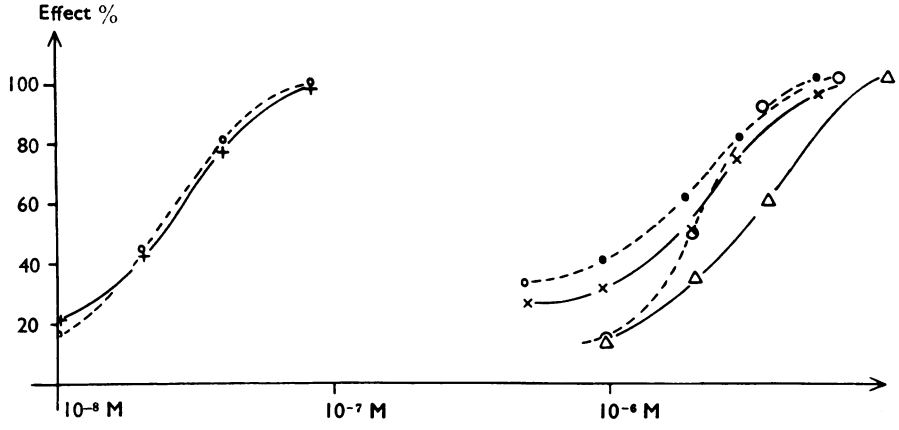

FIG. 3. Dose response curves for acetylcholine, isoprenaline and Ca^{2+} -ions. The curves were obtained in isolated atria, incubated either in circulating whole blood or in Tyrode solution. Each point on the curves represents the mean from at least three atria. +——+, ACh (blood); o--o, ACh (Tyrode); ×——×, Ca^{2+} (blood); ●--● Ca^{2+} (Tyrode); △——△, isoprenaline (blood); ○--○, isoprenaline (Tyrode).

in uptake for the different compounds studied. Moreover, no accumulation of the drugs by atrial tissue seems to take place, the apparent T/M ratios being between 0·4 and 1·1. In view of earlier observations on the uptake of the same drugs by atrial tissue, suspended in Tyrode solution these results seemed puzzling. In Tyrode

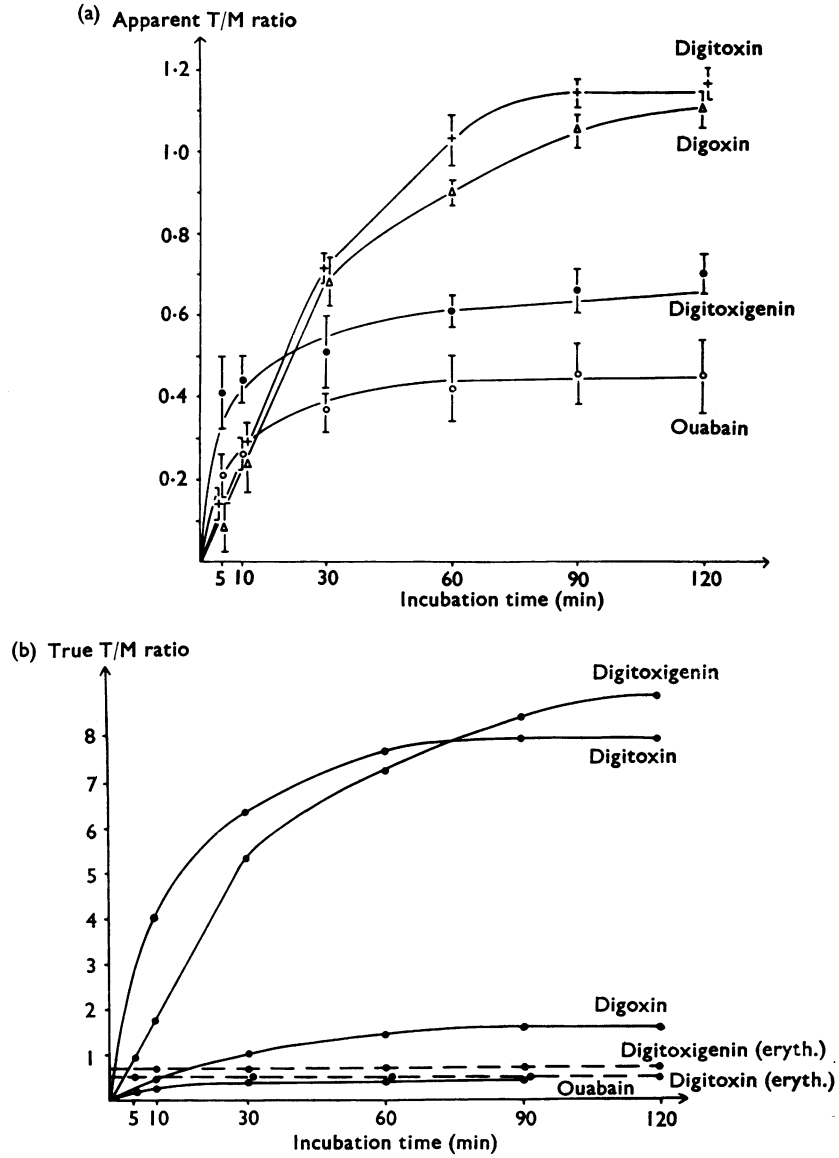

FIG. 4. a: Uptake of ^{3}H-ouabain ($9{\cdot}9 \times 10^{-8}$M), ^{3}H-digoxin ($3{\cdot}2 \times 10^{-7}$M), ^{3}H-digitoxin ($6{\cdot}5 \times 10^{-7}$M) and ^{3}H-digitoxigenin ($2{\cdot}2 \times 10^{-6}$M) by guinea-pig isolated atria, suspended in circulating, oxygenated whole blood. The atria were stimulated electrically (frequency 180/min). The "apparent" tissue/medium radioactivity ratio (T/M), was calculated from the total radioactivity in the serum of the circulating blood. b: Uptake of ^{3}H-labelled cardenolides by isolated atria, suspended in whole blood. Details as in Fig. 4a. In contrast to Fig. 4a, the "true" T/M ratio has been plotted as a function of the incubation time in Fig. 4b. For the calculation of this "true" ratio the amount of free, non-protein bound drug has been taken into account. The radioactivity in the erythrocytes is also shown.

solution, considerable differences had been observed between the uptake of ^{3}H-digitoxin and that of ^{3}H-ouabain: whereas ^{3}H-ouabain achieved a T/M ratio of only 0·5–0·6, ^{3}H-digitoxin showed a more than eight-fold accumulation with respect to the drug concentration in the aqueous medium (Kuschinsky, Lüllman & van Zwieten, 1968a). In whole blood, however, the binding of drugs to serum proteins should be taken into account. The degree of protein binding is known to be considerably different for the various cardenolides. Accordingly, the relative amount of free, non-protein bound drug also shows marked differences. The following values have been described for the concentration of the free, non-protein bound drugs (in % of the total amount): ouabain 100% ; digoxin 30% ; digitoxin 12% ; digitoxigenin 8% (Scholtan, Schlossmann & Rosenkranz, 1966 ; Kuschinsky, 1968).

In Fig. 4b, the uptake of the four different tritium-labelled drugs has been related to the concentration of the freely available compounds, taking the aforementioned data as a basis for the calculations. The curves shown in Fig. 4b reveal striking differences for the uptake of the different cardenolides. ^{3}H-digitoxin and also its aglycone now achieve an almost nine-fold accumulation, whereas ^{3}H-ouabain still only attains a low T/M ratio of approximately 0·5. ^{3}H-digoxin occupies an intermediate position in this respect. For the subthreshold bath concentration of digoxin the T/M ratio is about 1·6, for the toxic concentration about 1·0. Obviously, the accumulation of ^{3}H-digoxin is dose dependent. The T/M ratios calculated on the basis of the concentrations of free drug in the blood are in satisfactory agreement with those previously obtained in an entirely aqueous medium. The curves shown in Fig. 4b probably represent exponential functions. Upon application of the equation $Y = A\ (1\text{–}e^{-kt})$, where Y = uptake at time t, A = uptake at equilibrium, a plot of t versus ln $(A - Y)$ yielded straight lines. From the slope of these lines the rate constants (k) of the uptake processes could be calculated. In Table 2, the values for T/M, k and $t_{\frac{1}{2}}$ are summarized. The calculated data refer to the uptake curves in Fig. 4b.

Neither ^{3}H-ouabain nor ^{3}H-digoxin were taken up by the erythrocytes. As stated previously, the medium radioactivity was so high that even traces of the drugs would not have been missed. Both ^{3}H-digitoxin and ^{3}H-digitoxigenin, however, were taken up by the red cells, although to a lesser extent than by isolated atria. If, again, the concentration of the free drug was taken as a base for calculation (Fig. 4b), the T/M ratios achieved for ^{3}H-digitoxin and its aglycone in red cells proved to be about 0·5–0·7. These figures are considerably lower than those obtained in isolated atria for the same two drugs.

TABLE 2. *T/M ratios at equilibrium, rate constants (k) and half-lives of the uptake processes of ^{3}H-ouabain, ^{3}H-digoxin, ^{3}H-digitoxin and ^{3}H-digitoxigenin in subthreshold concentrations by isolated atria, suspended in circulating whole blood*

Compound	Medium conc. (M)	Apparent T/M ratio at equilibrium	"True" T/M ratios at equilibrium	k (sec^{-1})	$t_{\frac{1}{2}}$ (min)
Ouabain	$9{\cdot}9\times10^{-8}$	0·45	0·45	$1{\cdot}8\times10^{-4}$	6·5
Digoxin	$3{\cdot}2\times10^{-7}$	1·1	1·6	$7{\cdot}2\times10^{-5}$	16
Digitoxin	$6{\cdot}5\times10^{-7}$	1·1	8·85	$5{\cdot}4\times10^{-5}$	21·5
Digitoxigenin	$2{\cdot}2\times10^{-6}$	0·7	8·4	$2{\cdot}1\times10^{-5}$	5·5

The kinetic data k and $t_{\frac{1}{2}}$ given have been calculated in relation to the amount of non-protein bound, free drug in the blood (see Fig. 4b).

For none of the four ^{3}H-labelled cardenolides investigated could any significant concentration be detected in the buffer solutions, used for the washing of the erythrocytes (see **Methods**). The concentration of radioactive material was always much lower than that of the erythrocytes as such. It is evident, therefore, that the bound ^{3}H-labelled cardenolides cannot be easily washed out from the erythrocytes.

Discussion

Although the suspension of isolated organs in oxygenated whole blood would seem to be the best physiological method for studies on such preparations, to our knowledge, no suitable procedure has so far been described in the literature. The first approach to this problem was described by Vane (1964), who used oxygenated whole blood provided by larger laboratory animals. The method described in the present paper which employs whole blood circulating through an oxygenator seems suitable for pharmacological and other investigations on isolated organs over periods of several hours. The organs used in our experiments showed entirely normal mechanical activity, and the response to electrical stimuli and to drugs remained unchanged as compared with organs suspended in an aqueous medium. The studies in whole blood are limited, however, to organs consisting of relatively thin tissue layers, because otherwise the poor diffusion of oxygen would lead to insufficient oxygenation. As far as the response to drugs is concerned, the effect will be different from that in an aqueous medium if either protein binding, uptake by the erythrocytes or increased metabolic degradation occurs. Such was the case, for instance, with adrenaline. Various parameters of the circulating blood remained normal or at least physiologically acceptable. The haemoglobin content of the serum after 4 hr of circulation was 49 mg %, whereas in human cardiac surgery values of 70 mg% at the beginning of extracorporeal circulation are widely accepted. The pH of guinea-pig blood remained unchanged throughout the experiments. The value obtained in our experiments (7·8) is higher than that described in the literature (7·35, Spector, 1956), but a pH of 7·8 was repeatedly found, also in heparin-free blood obtained directly after killing the animals. This discrepancy between our results and those described in the literature remains unexplained. The oxygen saturation of the blood, circulating in our apparatus was optimal and as high as should be expected for arterial whole blood. Also the concentration of K^+, Na^+ and Ca^{2+} ions in serum remained normal throughout the experiments. No measurable loss of potassium from the erythrocytes occurred, possibly because the red cells were metabolically stabilized by the glucose solution, simultaneously infused.

The procedure proved suitable for kinetic studies on drugs in isolated atria, maintained in oxygenated whole blood. Detailed results have been described previously for kinetic studies with the same drugs in isolated atria, suspended in oxygenated Tyrode solution (Kuschinsky, Lahrtz, Lüllmann & van Zwieten, 1967; Kuschinsky, Lüllmann & van Zwieten, 1968a, b, c). As with the studies in Tyrode the uptake of the radioactively labelled drugs in blood reached equilibrium. The most striking difference was that an apparently low T/M ratio was obtained for ^{3}H-digitoxin and its genin, since both compounds showed a considerable cardiac accumulation in Tyrode. A quite different picture was obtained, however, if the amount of *free*, non-protein bound drug in the blood was taken as a basis for the calculations. Thus, at equilibrium ^{3}H-digitoxin and its genin showed a nine-fold

accumulation, whereas the T/M ratio for ^{3}H-ouabain remained as low as 0·5 (approximately). These results show satisfactory agreement with the T/M ratios obtained in Tyrode where protein binding cannot occur. The rate constants obtained in circulating whole blood as a medium also agreed with those previously obtained in atria, suspended in Tyrode solution, provided that the calculations are based on the amount of free, non-protein bound drug. From these results it may be concluded that the uptake of the cardiac glycosides and the aglycone is governed by the amount of freely available drug in the blood. The same probably holds true for the kinetic behaviour of cardiac glycosides *in vivo*. ^{3}H-ouabain and ^{3}H-digoxin were not taken up by erythrocytes, whereas a moderate uptake was observed for ^{3}H-digitoxin and its aglycone. Obviously the uptake of cardiac glycosides by erythrocytes increases with a decrease in polarity of the drugs in question. The lipid solubility of digitoxin and its genin is much higher than that of digoxin or ouabain (Waldi, 1962). Possibly, digitoxin and digitoxigenin combine with or dissolve in lipid constituents of the erythrocytes.

The use of functioning isolated organs suspended in circulating whole blood allows a closer approach to the conditions occurring in the living organism, whereas the advantages of isolated organ studies are still conserved: processes like elimination and non-specific uptake of the drug in tissues are avoided. The procedure described in the present paper has proved useful for kinetic studies on the distribution of cardiac glycosides. The results of these studies are in satisfactory agreement with data obtained in similar investigations, carried out in an aqueous medium. The procedure of incubation in circulating whole blood may well be extended to other isolated organs.

We are greatly indebted to Dr. S. Bloomfield (Burroughs Wellcome Inc., Tuckahoe, New York) and to Dr. H. Nowak (Merck AG., Darmstadt) for generous gifts of ^{3}H-digoxin and ^{3}H-digitoxigenin, respectively.

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(*Received December* 5, 1968)