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ADRIAMYCIN INDUCED CHANGES IN TRANSLOCATION OF SODIUM IONS IN TRANSPORTING EPITHELIAL CELLS

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Summary

The antitumor, antibiotic, adriamycin stimulates the net transport of sodium ions across frog skin epithelium under short circuit conditions. This stimulation is largely independent of Ca^{++} concentration in the media or of previous treatment of the epithelium with amiloride, ouabain, and vasopressin. We believe adriamycin induces changes in membrane permeability to sodium ions and that such changes may explain, in part, the cardiotoxicity of this drug.

The anthracyclic, antibiotic, adriamycin is an inhibitor of neoplasms, but its clinical use in chemotherapy has been limited owning to its cardiotoxicity (1-3). Adriamycin treatment leads to morphological damage in cardiac muscle of human patients (4) and of animal models (5). Biochemical lesions have been suggested but little direct evidence is available (6). Trump _et al_. (7,8,) have discussed changes in the pathophysiology of _cellular_ membranes resulting from perturbations by a variety of noxious agents and suggested that the physiological responses to cell injury manifested by morphological symptoms generally involve changes in membrane function.

Many antibiotics facilitate the translocation of ions across artificial and biological membranes (9-11). The action of a wide range of antibiotics on mitochondrial, erythrocyte and artificial phospholipid membranes is tentatively accounted for by changes in the passive permeability of the membrane to alkali and alkaline earth metal ions (10). Such studies to explore the altered electrochemical potential gradients, rates of transport, etc., have contributed much to the understanding of physiological transport processes. In particular, polar epithelial membranes have provided many fruitful experimental models (12).

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We have investigated the effects of adriamycin on frog skin, a convenient system in which active transport of sodium may be measured by determining the short circuit current (SCC) in the classical preparation of Ussing and Zerahn (13). The kinetic model of Curran et al. (14) was used to evaluate the kinetic parameters for sodium ion transport. This model assumes that sodium is the only ion transported and that the overall net transport of sodium ions across the epithelium is the algebraic sum of the fluxes across two significant barriers arranged in series. These barriers are the apical and basal surfaces of the transporting epithelial cells. The objectives of this report are to present the results of these sodium ion transport studies and to discuss briefly their potential significance in terms of the cardiotoxicity of adriamycin.

Materials and Methods

Drugs and Reagents

Adriamycin was obtained as a lyophilized powder from the National Cancer Institute. Vasopressin Grade IV, synthetic arginine vasopressin and ouabain were obtained from the Sigma Chemical Company. The amiloride was a gift generously provided by the Merck Sharp and Dohme Research Laboratory, Rahway, New Jersey.

The sodium Ringer's solution consisted of millimolar (mM): NaCl, 111.1; KCl, 2.40; $NaHCO_3$, 2.40; $CaCl_2$, 1.08; glucose, 5.5; the pH was 7.8 and the tonicity 225 milliosmolal (mOsm). The calcium and no-calcium Ringer's solutions consisted of (mM): NaCl, 92; KCl, 2: $NaHCO_3$, 2.4; glucose, 5.5; $CaCl_2$, 11.5 and NaCl, 92; choline chloride, 23; KCl, 2, $NaHCO_3$, 2.4, glucose, 5.5; EDTA, 0.15; respectively. The pH was 7.8 and the tonicity 225 mOsm.

Experimental Procedures

Frogs (*Rana pipiens*) were obtained from Mogul-Ed, Oshkosh, Wisconsin, and kept in cool running tap water prior to use. The frog was pithed and the abdominal skin removed, rinsed in the appropriate Ringer's solution and quickly mounted between lucite chambers as a flat sheet having an area of 3.16 cm^2 exposed to identical bathing solutions on both sides. Paired skins were obtained from the same frog by slitting the skin longitudinally along the abdomen to yield two pieces as nearly symmetrical as possible. The chambers employed were similar in design to those described by Sharp (16). They were equipped with two sets of electrodes. The open circuit potential differences across the skin was monitored with a pair of calomel electrodes (Radiometer K-401) in series with 3M KCl agar bridges using a Fluke Model 871A differential voltmeter. Current was passed through the skin from an external source by means of silver/silver chloride electrodes prepared according to methods described by Ives and Janz (17). A continuous recording of the short circuit current (SCC) was obtained by means of a simple voltage clamp circuit constructed with operational amplifiers. Both control and experimental short circuit currents were recorded on a Hewlett-Packard Model 7100B Dual-Pen Strip Chart Recorder.

After mounting, the skins were equilibrated with the appropriate Ringer's solution for ninety minutes prior to initiating the SCC. The bathing solutions were continuously aerated and mixed by slowly bubbling compressed air, that had been previously equilibrated with a large volume of buffer, through the solution. The total volume of solution in each chamber was 20 ml. The SCC was followed for 45-60 minutes prior to any treatment schedule. Most experiments were done with continuous SCC conditions following the open circuit equilibration period; however, occasionally intermittent short and open circuit conditions were used. Throughout these experiments the SCC has been taken as the net Na transport across the skin. Several previous investigators have shown this to be the case under control as well as under conditions of treatment with antidiuretic hormone (12). This conclusion was supported subsequently by isotope flux measurements using sodium-22. The adriamycin or other treatment drugs were added to the appropriate bathing solution as a concentrate with dilution yielding the desired experimental concentration.

All experiments were done at room temperature ($22^{0}C \pm 1^{0}$) with the temperature being monitored throughout the course of the experiment.

In experiments where the unidirectional flux of sodium was measured using sodium-22 isotope, the following procedures were followed. After the SCC had reached a steady value (usually 45-60 minutes following application of the voltage clamp), sodium-22 was added to the outside[1] bathing solution of one chamber assembly, A, and the inside bathing solution of the other chamber assembly, B, to make the final isotope concentration about 5 μCi per ml. Sampling of the inside bathing solution of chamber A began 2 minutes after injection of the isotope and continued for ten samples at 2-minutes intervals. After 60 minutes samples were taken at 10-minute intervals from the inside solution of chamber A, and also from the outside solution of chamber B. This latter sampling was continued for 60 minutes. One milliliter samples were withdrawn in each case and pipetted directly into 12 x 77 mm tubes for counting in a well-type scintillation counter (Nuclear-Chicago 1185 series Automatic Gamma Counting system). Immediately after sampling, the fluid volume in the chamber was restored by adding the appropriate Ringer's solution or Ringer's solution plus drug. At the end of the experiment an aliquot was taken from the outside solution of chamber A and the inside of chamber B, diluted appropriately and counted. The final volume of solution in each chamber was recorded. The skin was removed, washed for 30 seconds in fresh unlabeled Ringer's blotted on filter paper and placed directly in the counting tube to determine the total sodium-22 in the tissue exposed to the bathing solutions.

The detailed kinetic description of the model used and of the pertinent calculations are presented by Curran et al. (14) and by Clarkson and Lindemann (15).

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[1] Here outside refers to the side of the frog skin exposed to the external world. Inside refers to the serosal (body fluid) side of the frog skin.

Results

Effect of Adriamycin on Sodium Ion Transport Across Frog Skin

Adriamycin increases the net sodium ion transport across frog skin epithelium. Stimulation in SCC occurred approximately 4 minutes after addition of the drug and reached a quasisteady state value after 15-20 minutes. This enhanced SCC remained steady for up to 120 minutes, but more frequently began to fade slowly after 20-30 minutes at the maximum value. An enhancement ratio was defined as the maximum SCC divided by the initial SCC. Data from twenty experiments gave an average ratio of 1.5 ± 0.2 S.E. There were substantial differences in the open circuit potential and the initial short circuit current from frog to frog, but in all cases adriamycin treatment led to an increased SCC. This approximately 50% increase in the SCC will be shown later to be directly correlated to net sodium ion transport across the skin.

Dose response characteristics for acriamycin added to the inside bathing solution were such that a detectable response was obtained at 4×10^{-5}M. Saturation of the response occurred at approximately 5×10^{-4}M. If adriamycin were added to the outside bathing solution the first detectable response was at 8×10^{-5}M. This response showed saturation (ratio 1.2) at 8×10^{-4}M. However, a further addition of adriamycin to the inside bathing solution (4×10^{-4}M), so that both bathing solutions contained the drug, gave a further stimulation in the SCC (ratio 1.8). If the sequence of this treatment were reversed the stimulation on addition of the second dose was smaller. The delay of the response to adriamycin was shorter when the outside surface of the skin was treated (2-3 minutes versus 4-6 minutes) but the maximum response was always less than that obtained for inside surface treatment (1.2 versus 1.5 for the enhancement ratio).

The effect of adriamycin on the SCC was partially reversible on washing the treated skin with fresh Ringer's solution. With three washings the SCC returned to approximately 20% above the pretreatment value.

Effect of Adriamycin on Ouabain Treated Frog Skin

The effect of adriamycin treatment on the SCC of frog skin previously treated with ouabain is illustrated in Figure 1. Although the SCC is depressed substantially by the ouabain, adriamycin shows the simulatory effect (ratio 2.1) shown previously. If the skin were treated first with adriamycin (ratio 1.8) and then with ouabain, the response was characteristic of that shown in Figure 1. Ouabain has been shown to act on the Na^{+}/K^{+} ATPases involved in sodium transport (12) which are presumed to be in the basal-lateral surface membrane of the transporting epithelial cells in frog skin.

Effect of Adriamycin on Amiloride Treated Frog Skin

The effect of adriamycin treatment prior to or following treatment of the skin with amiloride is shown in Figure 2. Amiloride has been shown to act at the apical surface membrane in

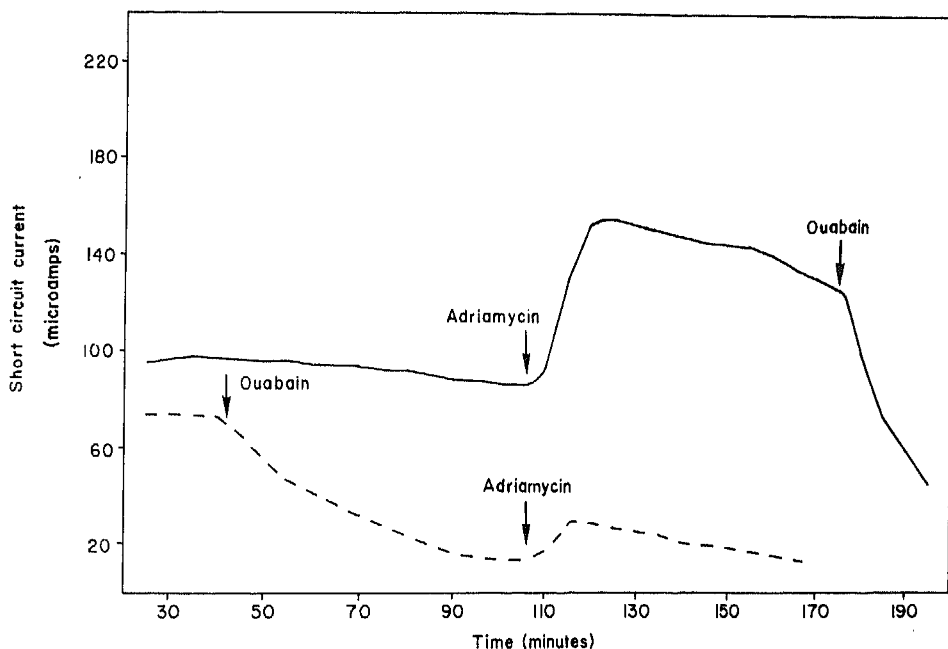

Fig. 1

A plot of short circuit current versus time illustrating the effect of treating frog skin epithelium with adriamycin (n=3). The dashed line illustrates the effect of adriamycin on skins previously treated with ouabain. The bathing solutions were Ringer's buffer, pH 7.8, 225 milliosmolal at 22 C. Following a ninety-minute open circuit equilibration period the skin was under continuous SCC conditions for forty-five minutes prior to starting treatment. Final concentration of the drugs was 10^{-5}M for ouabain and 4 X 10^{-4}M for adriamycin.

althering the transport of sodium and is readily reversible (18,19). Adriamycin stimulates SCC as effectively following the treatment with amiloride as prior to the treatment with amiloride. The action of amiloride is independent of whether the skins were previously treated with adriamycin. Experiments in which adriamycin and amiloride were added to the outside surface bathing solution showed no change in the pattern, but the stimulation by adriamycin was reduced, as in the control case for apical side treatments. These two drugs do not seem to compete for the same site of action in the sodium transport system of frog skin.

Effect of Adriamycin on Vasopressin Treated Frog Skins

The effect of adriamycin on vasopressin treated skins is illustrated in Figure 3. Vasopressin has been shown to act at the

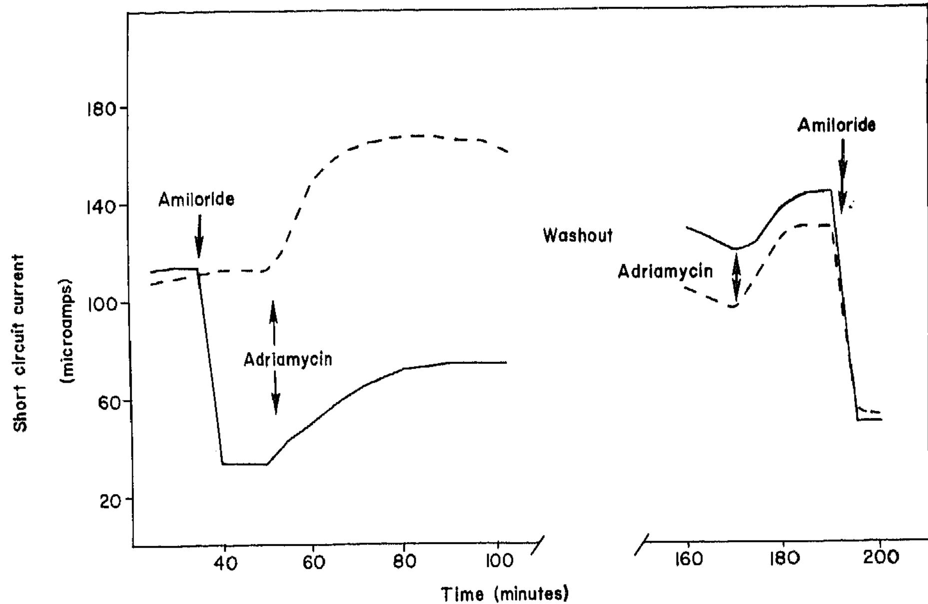

FIG. 2

A plot of SCC versus time illustrating the effect of adriamycin on frog skins treated previously with amiloride (1.2 X 10^{-5}M) (n=3). The Ringer's buffer had a pH, osmolality and temperature of 7.8, 225 mOsm and 22 C, respectively. Details of the equilibration procedures are given in the text. The solid line shows the results for the skin first treated with amiloride (1.2 X 10^{-5}M) on the outside, then followed by adriamycin (4 X 10^{-4}M) on the inside (serosal side). The dashed line indicates the control response. On rinsing three times with fresh Ringer's buffer the SCC returned to $\pm$ 20% of the pretreatment SCC. Subsequent treatment with adriamycin followed by amiloride is illustrated on the right side of the figure.

apical surface membrane to stimulate inward movement of sodium ions (14). Figure 3 shows the effect of vasopressin, when added to the inside bathing solution at a final concentration of 0.1 International Units per ml., along with the control curve. Adriamycin added to the inside bathing solution of both vasopressin treated and control skins produces the stimulatory effect.

Following a rinse (3X) of both chambers with fresh Ringer's solution, both control and experimental skins were treated with adriamycin (lateral-basal side), vasopressin (lateral-basal side), amiloride (apical side), and ouabain (lateral-basal side) in the sequence illustrated in Figure 3. Each drug shows its individual response in the presence of the other drugs. The treatment se-

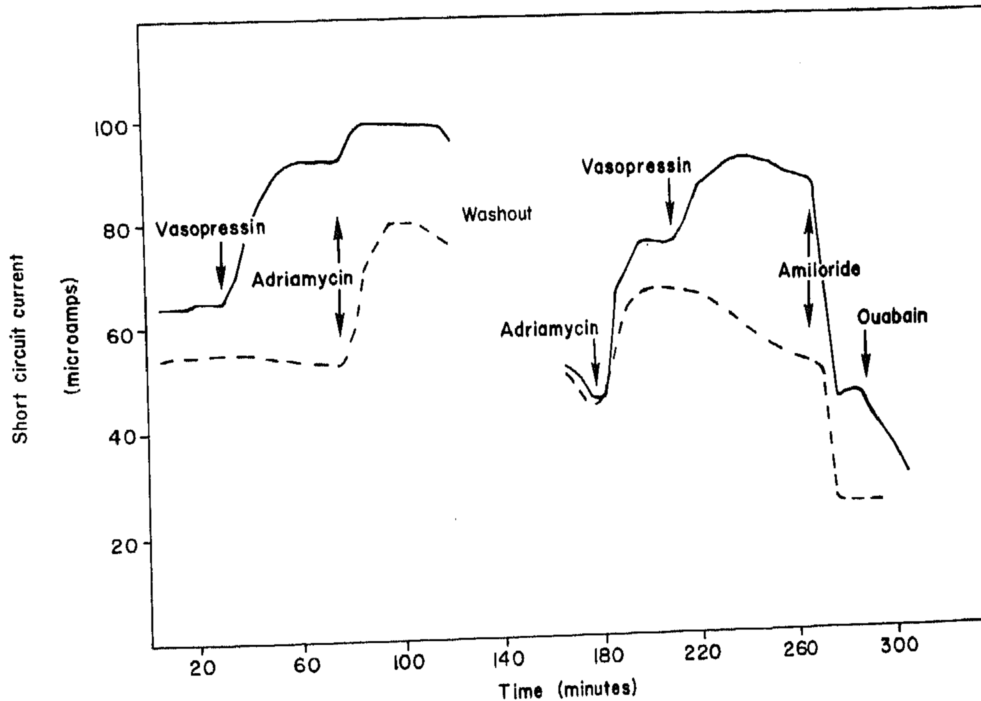

FIG. 3

A plot illustrating the effect of adriamycin on the SCC for frog skin previously treated with vasopressin (n=3). The pH, osmolality and temperature of the Ringer's buffer were 7.8, 225 mOsm and 22 C, respectively. See text for details of the equilibration procedures. The dashed line is the control and the solid line is the experimental curve with final concentrations of drugs as follows: vasopressin (0.1 International Units per ml), adriamycin (4×10^{-4}M), amiloride (1.2×10^{-5}M) and ouabain (4×10^{-5}M).

quence was of little consequence to the overall effect. These drugs apparently do not act at the same site in the sodium transport system of frog skin.

Effect of Adriamycin on Frog Skins Bathed in Calcium Rich Ringer's Solution.

Figure 4 illustrates the effect of adriamycin on the SCC for frog skins bathed in Ca^{++} rich Ringer's solution as contrasted with normal Ringer's containing 1.08 mM calcium ion. If the high calcium (11.5 mM) Ringer's solution were used as the bathing media on both sides of the skin, a lowered SCC was observed but the stimulatory effect of adriamycin was unaltered. Control experiments with varying Ca^{++} concentration from 0 to 11.5 mM gave results consistent with those reported by Herrara and Curran (20).

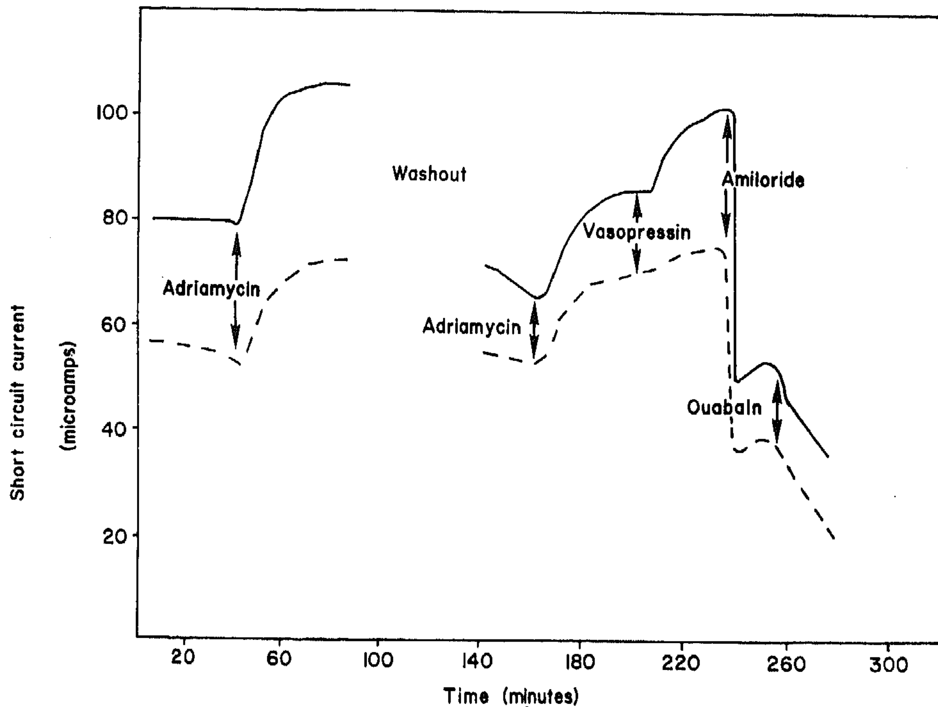

FIG. 4

Effect of adriamycin on the SCC for frog skin bathed in calcium rich Ringer's buffer (n=6). The pH, osmolality and temperature of the buffer were 7.8, 225 mOsm and 22 C, respectively. See text for details of the equilibration procedures. The solid line control curve had 1 mM Ca^{++} while the dashed line experimental curve contained 11.5 mM Ca^{++} with the appropriate reduction in NaCl to maintain the osmolality at 225 mOsm. Final concentrations of the drugs were: adriamycin (4 X 10^{-4}M), vasopressin (0.1 International Units per ml), amiloride (1.2 X 10^{-5}M) and ouabain (4 X 10^{-5}M).

The effect of adriamycin on the SCC was not significantly altered by varying the Ca^{++} concentration in the bathing solutions.

Following a rinse (3X) with the appropriate fresh Ringer's solution, the treatment sequence; adriamycin, vasopressin, amiloride, and ouabain was applied to both control and high calcium treated skins. The results are shown in Figure 4. Aside from the substantially reduced effect of vasopressin in the presence of high Ca^{++}, each drug showed its characteristic response. These results continue to support the tentative conclusion that these drugs have independent sites of action.

Effect of Adriamycin on the Kinetics of the Na Transport System in Frog Skin

The results of experiments to study the effects of adriamycin on the kinetics of the sodium ion transport system in frog skin are summarized in Table 1. The parameters listed in Table 1 are based on the kinetic model formulated by Curran *et al*. (14). The net transport of Na across the epithelium, given by J, increases 2.6 fold following adriamycin treatment. This result stems from three potential contributions: 1) greater Na permeability at the apical surface (P^0_{Na}), 2) a larger intracellular Na pool (S_2) and 3) an increase in the rate of Na transport across the basal surface (k_{23}). The greater value of k_{23} suggests that adriamycin acts directly on the active transport mechanism in frog skin. A similar kinetic analysis of vasopressin action by Curran *et al*. showed an increase in SCC and an increase in Na permeability of the outer membrane but no change in k_{23} (14).

TABLE 1.

Effect of Adriamycin on Sodium Ion Transport parameters for Frog Skin Epithelium

	J^A (μ eq-cm^{-2}-hr^{-1})	P^0_{Na} ^{B} (cm-hr^{-1})	k_{23} ^{C} (hr^{-1})	S_2 ^{D} (μ eq-cm^{-2})
Control	.99±0.04	0.067±0.005	3.7±0.05	0.17±0.01
Experimental	2.6 ±0.2	0.11 ±0.009	6.4±0.04	0.37±0.09

(A) J is the net sodium transport across the apical surface membrane and is equal to the net transport across the skin as measured by short-circuit-current in microequivalents per square centimeter of skin per hour. In each case the most probable error is indicated.

(B) P^0_{Na} is the sodium permeability of the apical surface membrane.

(C) k_{23} is the rate constant for unidirectional movement across the lateral-basal surface membrane.

(D) S_2 is the total sodium pool in the epithelium cell compartment. These values are actually subject to much greater uncertainties than suggested by the probable error listed (15).

Discussion

Adriamycin and Epithelial Transport

Although many aspects of the complex kinetics of ion passage through epithelial membranes have been studied (for recent reviews see Ussing and Thorn (21) and Finn (22)); it does not seem possible to give a strict mechanistic interpretation for the preliminary results presented in this paper. Despite this situation perhaps some of the effects observed may be useful in a qualitative sense.

Our main findings are that adriamycin stimulates the transport of sodium ion across the epithelium of frog skin independent of the effect of high concentration of calcium ion or of treatment with amiloride, ouabain and vasopressin. This effect of adriamycin on the translocation of sodium ions may result from either an increased availability of sodium ion to the active transport sites or from a direct stimulatory effect on the active transport mechanism itself. In this epithelium the asymmetric active transport of sodium ions is mainly controlled by the first living cell layer just under the stratum corneum (23). According to this two-barrier in series model the kinetics of transepithelial active transport of sodium are determined by rate limiting steps at the apical (outside) and basal-lateral plasma membranes of the transporting epithelial cells. The driving force for entry of sodium ion at the apical surface plasma membrane is presumed to be the simple electrochemical potential gradient (passive transport), whereas sodium ions are extruded across the basal-lateral membrane by the active transport mechanism. This model has been the basis for the interpretation of the action of compounds such as ouabain, amiloride, vasopressin, amphotericin B, etc., all of which modulate the transepithelial movement of sodium (12), and it serves here to interpret the effects of adriamycin.

The apparent lack of influence of amiloride, vasopressin or ouabain on the action of adriamycin suggests these drugs have independent sites of action. The change in passive permeability P^0_{Na} of the apical surface membrane suggests the primary action of adriamycin on transporting epithelial cells is to increase the passive transport across the apical surface membrane. The change in k_{23}, the rate constant for unidirectional transport across the basal-lateral surface membrane suggests a mild stimulation of the active transport mechanism or simply a change in the passive transport contribution to k_{23}. This observation, if it can be so interpreted, is in apparent conflict with other reports suggesting adriamycin inhibits Na-K ATPases (24) and other enzyme systems indispensible to the bioenergetics of the cell (25-27).

Voûte and Hänni (28) have shown that in an isoosmotic environment the total epithelial volume will depend on the rate of asymmetric active sodium transport in a positive and linear manner. Contributions to this volume increase arise from both the volume of the reactive epithelial cell layer and the volume of the extracellular space (29). The active transport system for Na^+ may have a major role in determining steady state cell volume under varying environmental conditions. Although there is no direct evidence that adriamycin produces these effects, it is plausible in light of other observations (2). Bucciarelli *et al.* (3) have found that adriamycin induces a nephrotic syndrome with massive proteinaria and edema in rats and suggest it could serve as a model for studying the pathophysiology of nephrotic proteinaria. Flamenbaum *et al.* (31) have suggested membrane dysfunction may be the primary mechanism of renal failure.

In summary, the primary cytotoxic effect of adriamycin on nondividing cells may arise from its tendency to disrupt the electrolyte balance of the cell.

Implications for Cardiotoxicity of Adriamycin

The results presented above may contribute to an understanding of the cardiotoxicity of adriamycin. Olson et al. (32) found that calcium and sodium concentrations were consistently higher in ventricular myocardial tissues from adriamycin-treated rabbits than from the controls. Thus, adriamycin seems to induce changes in the electrolyte balance of myocardial cells as well as epithelial cells. We will tentatively accept that adriamycin increases the passive influx of sodium ion into cells.

According to the hypothesis of Lehr (33) myocardial response to injurious agents involves an initial lesion of the myofibrils, including changes in the permeability of cellular and subcellular membranes. These permeability changes create electrolyte imbalances that indicate irreversible failure of cell function (7,8, 34). Lehr postulated that increased intracellular sodium initiates the process of myocardial cell degeneration and necrosis. This sequence of events parallels the action of adriamycin on transporting epithelial cells.

The initial increase in membrane permeability to sodium would increase the intracellular sodium, stimulating the metabolic extrusion of sodium. This response can compensate for the increased intracellular sodium ion concentration but at the expense of cell bioenergetics. In time, even a small increase in the passive permeability of the plasma membrane to sodium would lead to cell degradation and necrosis. This would be the net result even if adriamycin acted directly to stimulate the active transport system for sodium, a conclusion that cannot be ruled out by our results. If adriamycin inhibits ATPases, as suggested by Van Rossum and Gosalvez (24), cellular degeneration would be more rapid. The only difference in these two circumstances would be the relative capability of the perturbed cell to handle the increased intracellular sodium concentration and delay the loss of regulation of cell volume.

Jaenke (5) presented evidence of swelling in myocardial cells of adriamycin-treated rabbits. Further, these ultrastructural studies indicate myocardial cell mitochondria are severely affected by the adriamycin. A constant demand on the bioenergetics of the cell could result in accelerated "aging" of these organelles giving rise to the appearance of increased numbers of degenerating mitochondria, as observed in post mortem examinations of heart tissue from patients previously under adriamycin therapy (4). In contrast, adriamycin produced no effect on respiratory control rates or adenosinediphosphate to oxygen ratios in mitochondria isolated from control and adriamycin treated rabbits (6). This indicates the primary site of action for adriamycin is not the mitochondrion but perhaps, rather, the plasma membrane of myocardial cells. Recent evidence suggests that in mice this action may be associated with the peroxidation of membrane lipids (35).

It seems probable that a mode of action for adriamycin involving the plasma membrane may explain both the delayed (cardiomyopathic) and the acute (electrocardiographic) adriamycin induced myocardial toxicity (36). Acute adriamycin cardiac toxicity results from injection of large doses, and manifests itself in extreme tachycardia and/or myocardial arrest. This indicates a perturbation of the myofibril membrane depolarization, possibly

resulting from the adriamycin induced influx of sodium into the myocardial cells. An alternative explanation involving the release of endogenous catecholamines has been given for the antibiotic X537A (11); however, this explanation does not preclude a triggering of the process by altered sodium balance.

In conclusion we suggest that adriamycin induced changes in the electrolyte balance of myocardial cells may provide an approach for the interpretation of its cardiotoxicity.

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