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The effects of diphenylhydantoin and potassium on the biological activity of ouabain in the guinea-pig heart

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Summary

1. Diphenylhydantoin (DPH) and potassium significantly prevent ouabain intoxication without preventing the inotropic effects of ouabain in the guinea-pig isolated heart.

2. The antiarrhythmic effect of DPH and K on ouabain-induced toxicity appears to be related to their ability to reduce ouabain accumulation by the myocardium and thereby prevent the intracellular Na and K changes which lead to the arrhythmic state.

Introduction

The objective of this study was to compare the effects of diphenylhydantoin (DPH) and K on the accumulation of ouabain by the isolated heart which was reported by Dutta and his co-workers (Dutta, Goswami, Datta, Lindower & Marks, 1968a ; Dutta, Goswami, Lindower & Marks, 1968b ; Dutta & Marks 1969). The final goal was to investigate changes in the ionic status of the hearts in the presence of the selected agents, in order to determine if there were any relationships among the ability of these agents to prevent cardiac glycoside-induced arrhythmias, to prevent ouabain accumulation, and to alter the ionic status of the isolated perfused guinea-pig hearts.

The results suggest that diphenylhydantoin has the ability to reduce ouabain accumulation by the heart. This may be the pharmacological basis for the specific digitalis antagonism exhibited by diphenylhydantoin (see Mercer & Osborne, 1967 ; Scherlag, Helfant & Riciutti, 1968 ; Woodbury, 1969).

A preliminary account of some of this work has been given (Baskin & Dutta, 1970 ; Baskin & Dutta, 1971).

Methods

Isolated heart preparation

Male guinea-pigs, weighing 250–450 g were stunned by a sharp blow to the head. Their hearts were rapidly removed and placed in a vessel containing heparinized Krebs–Henseleit (Krebs) solution (Winegrad & Shanes, 1962) previously gassed with 95% O_2, 5% CO_2. The composition of the solution was: $NaHCO_3$ 27·2 mM, NaCl 118·0 mM, KCl 4·8 mM, KH_2PO_4 1·0 mM, $MgSO_4 \cdot 7H_2O$ 1·2 mM, $CaCl_2 \cdot 2H_2O$ 2·5 mM and glucose 11·1 mM. Hearts were mounted on a perfusion apparatus and perfused at 28° C with Krebs solution through the aorta at a rate of 4 ml/min by means of a roller pump.

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Effect of diphenylhydantoin (DPH) and elevated potassium on ^{3}H-ouabain accumulation

The effect of DPH and K on ^{3}H-ouabain accumulation was studied in the spontaneously beating heart. ^{3}H-Ouabain was supplied by the New England Nuclear Inc. at a specific activity of 11·7 Ci/mM. For these experiments, following an initial 64 min equilibration period, 1×10^{-7}M ^{3}H-ouabain was perfused for 64 min either alone, or in the presence of a second agent. No washout period was employed. The hearts were immediately removed from the perfusion apparatus, homogenized, and the ^{3}H-ouabain was analysed in homogenate and subcellular fractions by the methods of Dutta *et al.* (1968a).

Effect of diphenylhydantoin and elevated potassium on ouabain-induced arrhythmias

Two agents which antagonize ouabain-induced arrhythmias were studied. The hearts were stimulated at 1·25 Hz (5 ms duration, voltage 10% or less above threshold) after the elimination of atrial and nodal pacemakers according to the method of Benforado (1958). For these experiments ^{3}H-ouabain 5×10^{-6}M was perfused for 15 min either alone or in the presence of a second agent through hearts that previously had been equilibrated for the usual 64 min period. This concentration of ouabain invariably induced arrhythmias (extra beats) by the end of the 15 min period. In these hearts a Brush transducer was used to record displacement as an index of mechanical activity. The first derivative of displacement was obtained electronically and was used as a measure of inotropy.

Analysis of sodium, potassium and calcium

An aliquot of homogenate was placed in a plastic tube with an equal volume of 14 N nitric acid containing 2% lanthanum. After 24 h the digest was heated slowly to 100° C, maintained for 1 h, allowed to cool to room temperature and reconstituted to the original volume. Na and K were analysed by flame emission spectrophotometry and Ca by atomic absorption spectrophotometry.

Accumulation of ^{22}Na and ^{45}Ca

Radioactive sodium and radioactive calcium were perfused in Krebs medium for 15 min through guinea-pig hearts as described above. Radioactive sodium (^{22}Na) was obtained carrier free from International Chemical and Nuclear Inc. Aliquots of heart homogenates were analysed with a Packard auto-γ-spectrometer. Radioactive calcium (^{45}Ca) was also purchased from International Chemical and Nuclear Inc. Radioactive calcium content of heart homogenate aliquots was measured in a liquid scintillation spectrometer, with Bray's scintillation solution (Bray, 1960). Corrections were made for radioactive decay.

Analysis of interstitial space

The volume of the interstitial space was determined with inulin as a marker (Tuttle, Witt & Farah, 1961). The method of Schreiner (1950) was used for the measurement of inulin.

Intracellular ion concentration, was derived as follows, using K for illustrative purposes:

$$[K]i=(Kt-Vin\ [K]o)/(V-Vin)$$

in which

[K]i=the intracellular K concentration

[K]o=the K concentration of the medium

Kt=the total K content of the muscle

Vin=the volume of the inulin space

V=the volume of the total tissue water derived by wet weight and dry weight measurement and assuming specific gravity of 1·0.

No assumptions were made as to the physical state of the intracellular Na, K or Ca.

By the use of procedures adapted from Tuttle *et al.* (1961), the inulin space was found to be 29·7% as seen in Figure 1. The hearts were perfused with 1% inulin in Krebs and inulin concentrations were determined at the indicated time intervals.

The total water concentration was found after perfusion to be 79·0% ±5·5 by measuring wet and dry weight samples. This value was not different under conditions in which DPH and elevated K were examined for their ability to alter ouabain inotropy and arrhythmia.

All statistical analyses were made using Student's *t* test (Snedecor, 1956). *P* values greater than 0·05 were not considered significant.

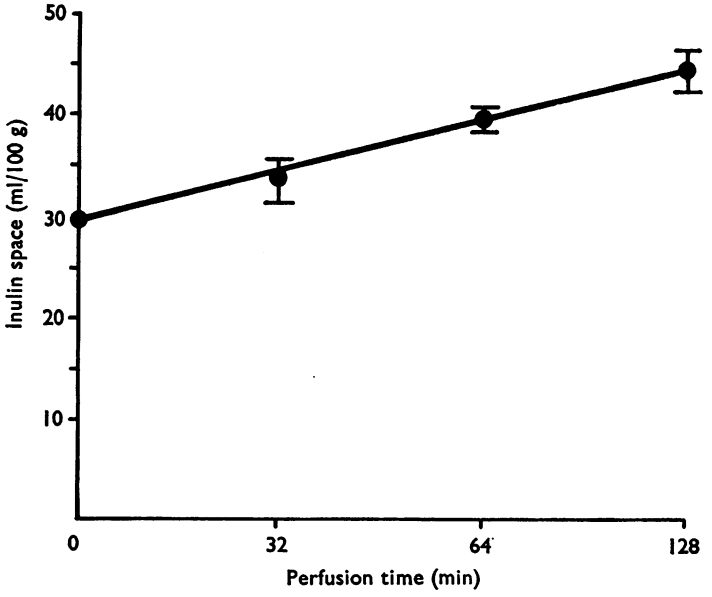

FIG. 1. The extracellular space as determined by inulin at different time intervals in guinea-pig heart. Hearts were perfused with Krebs containing 1% inulin for the different time intervals at 28° C with a flow rate of 4 ml/minute. The percent of inulin solution in ventricular tissue is shown on the ordinates. Time of incubation in inulin solution is shown on the abscissae. Standard errors are represented by vertical lines through each point. Each point is the mean of at least three values.

Results

Effects of diphenylhydantoin and potassium on ^{3}H-ouabain accumulation

In the spontaneously beating isolated guinea-pig heart, diphenylhydantoin (1×10^{-4}M) and 2× normal potassium caused significant inhibition of ouabain accumulation (Table 1A). Table 1B shows the accumulation of ^{3}H-ouabain alone and in the presence of 6×10^{-5}M and 1×10^{-4}M DPH respectively and 1·5× normal potassium. Diphenylhydantoin reduced the accumulation of ouabain by the myocardium. Potassium also produced a small but significant reduction in the accumulation of ouabain. Concentrations of DPH and K which reduced the accumulation of ouabain also prevented the ouabain-induced arrhythmias. The effect of DPH and K on the subcellular distribution of ouabain was also analysed. Table 2 illustrates that DPH and twice normal K which reduced homogenate concentrations of ^{3}H-ouabain similarly reduced all the particulate fractions. The supernatant-to-particulate ratio (S/P) was increased, indicating that the affinity of ouabain for membrane binding sites was reduced both by DPH and K.

TABLE 1A. *Effect of diphenylhydantoin and potassium on the accumulation of an inotropic concentration of ouabain**

Agent	^{3}H-Ouabain	Accumulation (pmol/g wet wt.)
Ouabain 1×10^{-7}M	(7)†	182·5± 7·5
+Potassium ions 2× normal	(3)	89·6±11·0 $P<0·001$
+Diphenylhydantoin 1×10^{-4}M	(4)	82·7± 2·0 $P<0·001$

* Spontaneously beating hearts were equilibrated for 64 min with K-H medium, then perfused for 64 min with labelled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28° C, stimulated at 1·25 Hz. Values indicate mean ± S.E.
† Number of experiments.

TABLE 1B. *Effect of diphenylhydantoin and potassium on the accumulation of an arrhythmogenic concentration of ouabain**

Agent	^{3}H-Ouabain	Accumulation (pmol/g wet wt.)
Ouabain 5×10^{-6}M	(6)†	2980± 59
+Diphenylhydantoin 6×10^{-5}M	(3)	2621±116 $P<0·05$
+Diphenylhydantoin 1×10^{-4}M	(4)	2298±192 $P<0·05$
+Potassium 1·5×normal	(3)	2708± 25 $P<0·05$

* Hearts were equilibrated for 64 min with K-H medium, then perfused for 15 min with labelled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28° C, stimulated at 1·25 Hz. Values indicate mean ± S.E.
† Number of experiments.

TABLE 2. *Distribution of ouabain in subcellular fractions of hearts perfused with anti-arrhythmic drugs**

Agents	^{3}H-Ouabain (pmol/mg protein) *Nuclear*	*Mitochondrial*	*Microsomal*	S/P ratio†
Ouabain 1×10^{-7}M (7)‡	0·75±0·06	0·80±0·09	1·51±0·18	1·13±0·22
+2×potassium (3)	0·23±0·04 $P<0·001$	0·39±0·21 NS§	0·39±0·01 $P<·001$	5·87±2·41 $P<0·05$
+Diphenylhydantoin 1×10^{-4}M (4)	0·31±0·08 $P<·005$	0·23±0·03 $P<0·001$	0·53±0·17 $P<0·005$	2·87±0·28 $P<0·001$

*Spontaneously beating hearts were equilibrated for 64 min with K–H medium, then perfused for 64 min with labelled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28° C. Values indicate mean ± S.E.
†Ratio of dpm in supernatant to pellet fraction after centrifugation of heart homogenate at 100,000 × g for 60 minutes.
‡Number of experiments.
§NS=not significant.

Effects of diphenylhydantoin and elevated extracellular potassium on the ability of ouabain to produce inotropy and arrhythmia

Figure 2 illustrates three representative experiments showing the effects of 5×10^{-6}M ouabain on the mechanical activity of the paced heart. As shown in the top panel, positive inotropy was seen within 2–4 min, and gradually reached its maximum at 9 minutes. Following that period there was an onset of arrhythmia as indicated by the presence of extra beats which continued until the end of the experiment at 15 minutes.

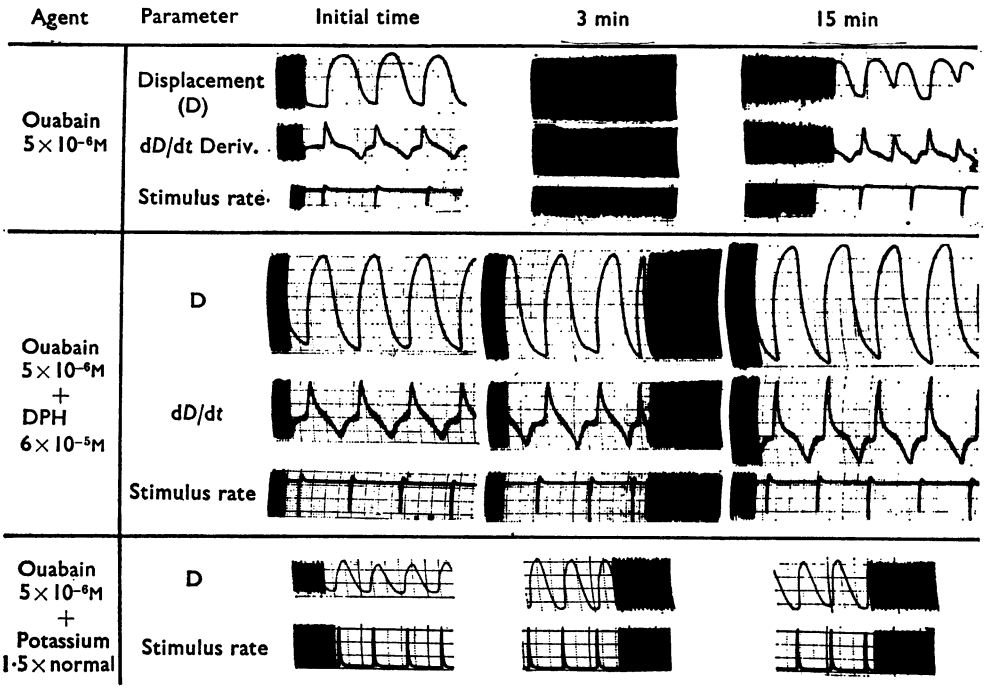

FIG. 2. Effect of diphenylhydantoin and potassium on the positive inotropic and arrhythmic actions of ouabain. Guinea-pig hearts were equilibrated for 64 min with Krebs medium, then perfused for 15 min with various substances as indicated above. Flow rate was 4 ml/min and the temperature was 28° C. The hearts were stimulated at 1·25 Hz (5 ms duration).

The middle panel shows that in the presence of 6×10^{-5}M DPH, ouabain was still able to exert its positive inotropic effect. However, DPH prevented the production of extra beats as shown in the right-hand side of the middle panel. These findings are comparable to earlier observations made by Helfant, Scherlag & Damato (1967) in the intact dog.

The bottom panel shows that in the presence of 1·5 × normal K, ouabain was able to exert its positive inotropic effect. However, K prevented the onset of extra beats in this time period, as was observed with the administration of DPH. These findings are in agreement with those of Williams, Klocke & Braunwald (1966).

It has been reported by earlier workers (Drake, Haury & Gruber, 1939 ; Covino & Shannon, 1969) that DPH decreases the contractility of muscle. Figure 3 shows that increased DPH caused dose-dependent depression of heart contractility as

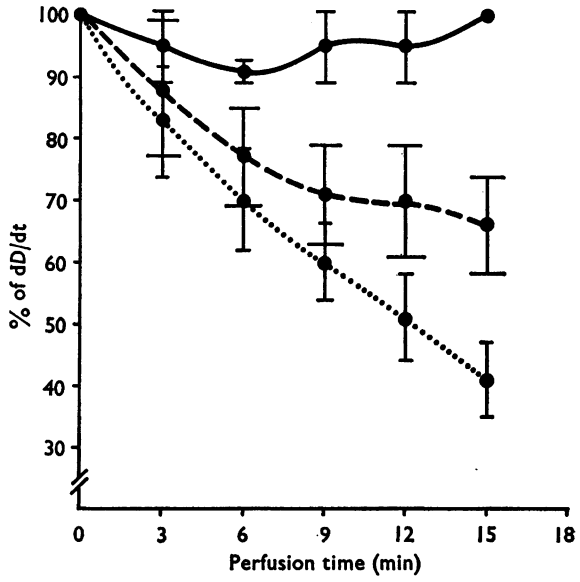

FIG. 3. Diphenylhydantoin (DPH) time-response relationships. Percent of dD/dt at zero time is shown on ordinates. Perfusion time is shown on the abscissae. Standard errors are represented by vertical lines intersecting each point. Each point represents at least 5 values. Control—unbroken line; in presence of DPH 6×10^{-5}M—broken line; in presence of DPH 1×10^{-4}M—dotted line.

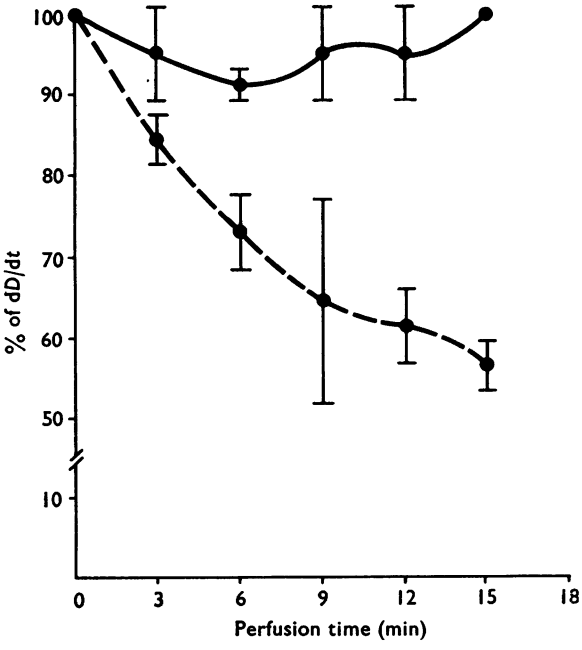

FIG. 4. 1·5× Normal potassium time-response relationship. Percent of dD/dt at zero time is shown on ordinates. The perfusion time is shown on the abscissae. Standard errors are represented by vertical lines through each point. Each point represents 4 values. Control—unbroken line; in presence of 1·5× normal K—broken line.

measured by the first derivative of the displacement of an isotonic lever (dD/dt). In 15 min the velocity of shortening was reduced by more than 50% by 1×10^{-4}M DPH. Also, 1·5 × normal K depressed cardiac contractility with a similar time-effect relationship (Figure 4).

Although DPH and 1·5 × normal K had a depressant effect upon the guinea-pig heart, they did not eliminate the positive inotropic response observed when ouabain, in a concentration of 5×10^{-6}M, was perfused for 15 minutes. Instead, it appears that these agents only increased the time required for the maximum response to develop, without altering the magnitude of that maximum response (Fig. 5 and 6). The same figures also show the time course of the onset of arrhythmias (Cross-hatched lines, in ouabain-perfused hearts 12 ± 2 min), and indicate that in none of the DPH or 1·5 K-perfused hearts did an arrhythmia develop.

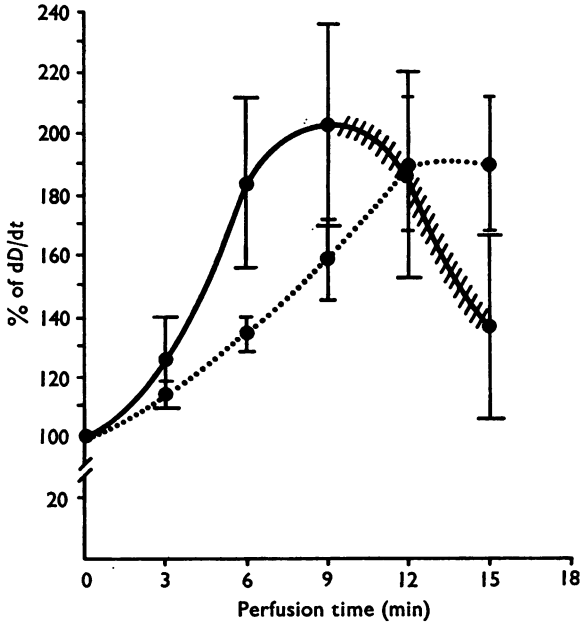

FIG. 5. Ouabain/diphenylhydantoin time-response relationships. Percent of dD/dt at zero time shown on ordinates. Perfusion time is shown on the abscissae. Standard errors are represented by vertical lines intersecting each point. Each point represents at least 3 values. Solid line—in presence of ouabain 5×10^{-6}M. Dotted line—in presence of 1·5× normal K plus ouabain 5×10^{-6}M. The hatched area represents the presence of cardiac arrhythmia.

Effects of diphenylhydantoin and 1·5× normal K on intracellular ion concentrations

In the same hearts in which the accumulation of ouabain was studied, intracellular ion concentrations were also examined. In agreement with many other studies (see Glynn, 1964), Table 3 shows that an arrhythmic concentration of ouabain increased the intracellular Na ion concentration. The addition of either DPH or 1·5× normal K prevented the increase in the intracellular Na ion concentration. It should be noted that in the presence of DPH alone (6×10^{-5}M or 1×10^{-4}M) there was only an insignificant diminution in the intracellular Na ion concentration.

Although DPH alone failed to alter significantly the intracellular Na ion concentration, it did exhibit a dose-dependent effect on the exchange of ^{22}Na in the myo-

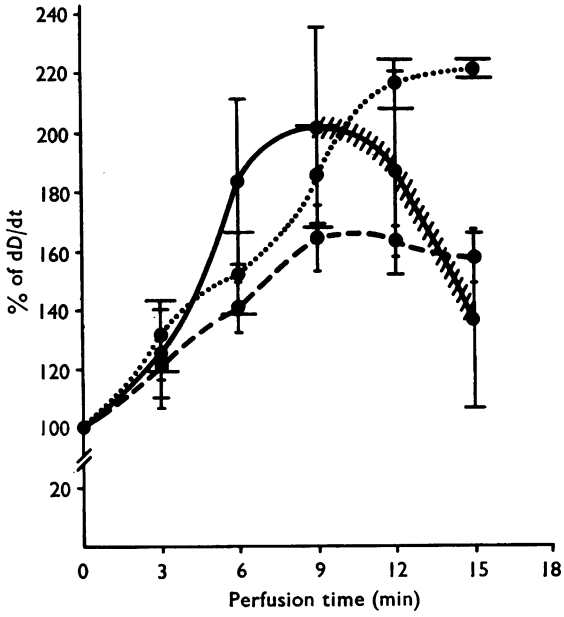

FIG. 6. Ouabain/1·5× normal potassium time response relationships. Percent of dD/dt at zero time shown on ordinates. Perfusion time is shown on the abscissae. Standard errors are represented by vertical lines intersecting each point. Each point represents at least 4 values. Solid line—in presence of ouabain 5×10^{-6}M. Broken line—in presence of ouabain 5×10^{-6}M plus diphenylhydantoin 6×10^{-5}M. Dotted line—in presence of ouabain 5×10^{-6}M plus diphenylhydantoin 1×10^{-4}M. The hatched area represents the presence of cardiac arrhythmia.

TABLE 3. *The effect of ouabain, diphenylhydantoin and extracellular potassium on intracellular ion concentrations in guinea-pig hearts**

Agent		Intracellular Na mEq/l.H_2O	Intracellular K mEq./l.H_2O	Intracellular Ca mEq/l.H_2O
Control	(4)†	43·9±2.5	139·2±3·4	3·83±0·30
Ouabain 5×10^{-6}M	(5)	59·8±1·2 $P<0·001$	120·1±4·5 $P<0·025$	3·15±0·12 NS‡
+Diphenylhydantoin 6×10^{-5}M	(3)	42·0±4·8 $P<0·025$§	138·7±4·7 $P<0·05$§	3·48±0·25 NS
+1·5× normal potassium	(4)	39·2±2·4 $P<0·001$§	132·7±2·7 $P<0·05$§	3·82±0·26 NS
Diphenylhydantoin 1×10^{-4}M	(4)	38·2±7·1 NS‡	134·0±7·0 NS‡	7·60±1·10 $P<0·05$
Diphenylhydantoin 6×10^{-5}M	(3)	41·2±9·6 NS	137·6±5·8 NS	3·57±0·34 NS

*Hearts were equilibrated for 64 min with Krebs medium, then perfused for 15 min with labelled ouabain either alone or with the various substances indicated. Flow rate 4 ml/min, temperature 28° C, stimulated at 1·25 Hz. Values indicate mean ± S.E.
†Number of experiments.
‡NS=not significant.
§With reference to ouabain as control.

cardium (Table 4). Both DPH and 1·5× normal K significantly increased ^{22}Na exchange under the same conditions in which the cellular sodium content was unaltered.

Table 3 shows the effects of DPH and K on the changes in intracellular K concentration produced by ouabain. An arrhythmic concentration of ouabain decreased intracellular K concentration significantly while the addition of either DPH or 1·5× normal K prevented the reduction in intracellular K. In the absence of ouabain, DPH appeared to exert little effect on the intracellular K concentration.

The effect of an antiarrhythmic concentration of DPH and 1·5× normal K on the intracellular Ca ion concentration is shown in Table 3. Within the 15 min

TABLE 4. *The effect of diphenylhydantoin and 1·5× normal potassium on ^{22}Na accumulation**

Agent		Intracellular ^{22}Na accumulation mEq/l.H_2O
Control	(5)†	19·3±1·7
Diphenylhydantoin 3×10^{-5}M	(3)	28·9± 0·1 $P<0·005$
Diphenylhydantoin 6×10^{-5}M	(3)	33·7± 4·7 $P<0·025$
Diphenylhydantoin 1×10^{-4}M	(5)	38·7±12·0 NS‡
1·5× normal potassium	(5)	36·6± 4·7 $P<0·01$

* Hearts were equilibrated for 64 min with Krebs medium, then perfused for 15 min with the various substances indicated. Flow rate 4 ml/min, temperature 28° C, stimulated at 1·25 Hz. Values indicate mean ± S.E.
†Number of experiments.
‡NS=not significant.

time period of the experiment there were no significant changes in the intracellular Ca concentration except for hearts perfused with a high concentration of DPH alone. The special action of this high concentration of DPH on calcium balance was further substantiated in experiments on calcium exchange, using ^{45}Ca (Table 5). This dose of DPH significantly increased ^{45}Ca exchange. No other treatments were effective.

TABLE 5. *The effect of diphenylhydantoin on ^{45}Ca accumulation in isolated perfused guinea-pig hearts**

Agent		Intracellular ^{45}Ca accumulation mEq/l.H_2O
Control	(3)†	3·56±0·18
Diphenylhydantoin 1×10^{-5}M	(3)	3·74±0·13 NS‡
Diphenylhydantoin 3×10^{-5}M	(3)	4·17±0·26 NS
Diphenylhydantoin 6×10^{-5}M	(3)	3·95±0·20 NS
Diphenylhydantoin 1×10^{-4}M	(3)	5·53±0·40 $P<0·05$

* Hearts were equilibrated for 64 min with Krebs medium, then perfused for 15 min with the various substances indicated. Flow rate 4 ml/min, temperature 28° C, stimulated at 1·25 Hz. Values indicate mean ± S.E.
†Number of experiments.
‡NS=not significant.

Discussion

These experiments show that: (1) DPH and K have the ability to reduce ouabain accumulation by the guinea-pig heart; (2) DPH and K also prevent ouabain-induced arrhythmia; (3) DPH and K prevent the ionic changes produced in heart muscle by ouabain; (4) DPH and K delay the development of the inotropic action of ouabain. These results suggest that agents which reduce ouabain accumulation may prevent ouabain intoxication.

DPH and elevated K reduced the accumulation of ouabain by the heart. The effects of K on reducing ouabain binding (Dutta & Marks, 1969) and preventing ouabain intoxication (Sampson, Albertson & Kondo, 1943) have been reported. Although DPH is believed to specifically antagonize digitalis arrhythmias (see Mercer & Osborne, 1967; Sherlag *et al.*, 1968) the effect of DPH on ouabain binding is not known. There are at least two compartments for ouabain binding. Ouabain given in a low concentration, primarily occupies the saturable (specific)

compartment of ouabain binding sites, while a high concentration of ouabain primarily occupies the non-saturable, non-specific compartment (Dutta *et al.*, 1968b). Therefore, the marked effect of DPH and K seen with a low dose of ouabain appears to indicate a reduction of the specific ouabain binding. The small reduction in ouabain binding after perfusion with a large dose of ouabain indicates a lack of effect of DPH and K on the large non-saturable, non-specific compartment. In addition, the subcellular fractionation pattern of ouabain remained consistent for those hearts treated with DPH or with elevated K. The concentration of ^{3}H-ouabain was found to be greater in the microsomal fraction than in any other fraction of the heart homogenate. With DPH and with high K, however, there was significant reduction in particulate binding and an elevation of supernate/pellet ratio.

As a result of the preliminary binding study under conditions in which ouabain (1×10^{-7}M) did not exhibit arrhythmic properties, DPH and K were chosen for further investigation in experiments designed to test their ability to prevent ouabain toxicity. The experimental data indicate that the production of a positive inotropic response by ouabain is independent of the negative inotropic response produced by DPH and K. However, DPH and K did prevent the production of arrhythmias during this period. This anti-arrhythmic effect is associated with a concomitant reduction in ouabain accumulation, and the prevention of ouabain-induced increase in intracellular Na and decrease in intracellular K. These observations are not in agreement with those of Godfraind, Lesne & Pousti (1971) who found no reduction in ouabain binding with DPH. Since the tissue and experimental conditions (most notably, temperature) were different it is difficult to compare experiments.

Scharff, Lautier, Laverenne & Duchene-Marullaz (1969) showed that in the denervated dog, unlike the intact animal, DPH decreased contractile activity. In the isolated heart, DPH also appears to depress contractile activity, which is in agreement with other isolated preparation studies (Drake *et al.*, 1939 ; Covino & Shannon, 1969).

Analysis of the time-course of the ouabain effect on myocardial contractility (isotonic displacement) suggests that DPH and K both caused a delay in the production of the positive inotropic response to ouabain. However, at later time periods, the magnitude of the inotropic response in the presence of DPH may be equal to or greater than that of ouabain alone. This could be due to a slower rate of ouabain accumulation by the heart under these conditions along with the observed reduction in total ouabain uptake, but data on the influence of DPH and K on the rate of ouabain uptake are not yet available. The results of this study would suggest that the effect of DPH and K on the positive inotropic response of ouabain should be examined under conditions in which only positive inotropy and not arrhythmia is present. Apparent increases in ouabain inotropy due to DPH and K measured near the time of ouabain toxicity (see Scherlag *et al.*, 1968 ; Williams *et al.*, 1966) may be misleading if the enhancement in inotropy seen in the presence of the antiarrhythmic agents is thought to occur at all time periods of drug interaction.

Although DPH increased sodium exchange in heart muscle without changing intracellular sodium concentration, it would be premature to regard this as evidence for direct stimulation of Na-K-ATPase. Recent conflicting findings of Danielson, Bittar, Chen & Tong (1971) who found that DPH inhibited Na efflux in barnacle

muscles, and Lewin & Bleck (1971) who showed that DPH can stimulate kidney Na-K-ATPase, emphasize that no clear pattern regarding DPH has emerged. The increased intracellular ^{22}Na with the addition of DPH shows that DPH increases the membrane permeability to Na. These data and those of Watson & Woodbury (1972) would not support the hypothesis of Sano, Suzuki, Sato & Ida (1968) and Lüllman & Weber (1968) that DPH inhibits Na influx into the myocardium.

Although changes in intracellular Na and K concentrations may be due in part to the arrhythmia itself, ouabain may also cause the changes in intracellular Na and K concentrations. The inhibitory effect of ouabain on ion transport is well documented (see Glynn, 1964). Since DPH and K decrease ouabain accumulation at both inotropic and arrhythmic concentrations, the prevention of ion changes may be due to the decrease of the ouabain concentration.

We find that, in high doses, DPH increases the uptake of Ca, which would agree with the studies of Blaustein (1967). However, these changes could not be compatible with the antiarrhythmic properties of DPH since the antiarrhythmic effects were observed at a DPH concentration in which calcium changes were not observed.

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