Induction of Failure in Gas-Perfused Hearts by Intermittent Administration of Krebs Solution

THE EFFECT OF DIGITALIS GLYCOSIDES
By Lloyd P. Gobel, Ph.D., Ivan Bihler, Ph.D., and Peter E. Dresel, Ph.D.

ABSTRACT
Our previous work has shown that kitten hearts perfused with a warm, moist mixture of 5% CO₂-95% O₂ beat strongly for many hours. We now show that after 1 hr of gas perfusion, intermittent administration of small volumes of Krebs solution (5 ml every 30 min to total of 20 ml) causes progressive failure of the heart. Washout cardiac failure is accompanied by loss of protein from the hearts. We have isolated a cardiotonic material from the washout liquid by precipitation with ammonium sulfate. This material is more active in failed than in normal isolated kitten atria. Preperfusion with digitalis glycosides (10⁻¹⁰ to 10⁻⁹ g/ml) protects hearts against failure and loss of protein during periods of washout with saline.

ADDITIONAL KEY WORDS
contractile force
inotropic substances
adenylates
removal of protein
cat hearts

We have recently described some of the properties of small cat hearts perfused with a warm, moist mixture of 95% O₂-5% CO₂ (1). Gas-perfused hearts beat more strongly and fail much more slowly than do hearts perfused with substrate-free Krebs solution. An obvious difference between gas- and liquid-perfused Langendorff hearts is that the gas mixture cannot remove any nonvolatile materials from the preparation. Clark et al. observed that recirculation of a liquid perfusion medium lengthened the period of adequate contractility of frog hearts and indicated that failure might be due to the loss of an active substance from perfused preparations (2). The present work was undertaken to determine whether the prolonged viability of gas-perfused hearts might be due to prevention of such loss. Our results are compatible with this hypothesis and demonstrate that extremely low concentrations of cardiac glycosides inhibit the loss of this material.

Method
A complete description of our method for perfusing the hearts of kittens with gas mixtures has been published (1). Hearts weighing 4 to 10 g were perfused through the aorta first with substrate-free Krebs solution for 5 min ("preperfusion") and then with 95% O₂-5% CO₂. Care was taken to maintain the temperature (37.5 ± 0.5°C) and humidity (100%) of the perfusion media and of the plastic box containing the hearts. The heart rate was kept constant at 168 beat/min by suprathreshold electrical stimuli (5 msec, 2 to 10 v) from a Grass SD5 or SS stimulator, the electrodes being attached to the right atrium and the ventricular apex. Contractile force was measured with a Grass FT-03 force-displacement transducer connected to a hook through the ventricular apex. Recordings were made with a Grass Polygraph.
Five milliliters of warm oxygenated Krebs solution was injected at specified times through a fine polyethylene tube inserted into the perfusion cannula. Care was taken during the injection to prevent formation of multiple gas-liquid interfaces that would interfere with the perfusion. Passage of the liquid through the heart was complete in 20 to 30 sec. The injected liquid dropped through a funnel, was collected in graduated test tubes and was brought back to its original volume of 5 ml by addition of Krebs solution (less than 0.5 ml was required in all instances); a 0.5-ml sample was removed for analysis of protein and adenylic acid and the remainder was saturated to 25% with dry ammonium sulfate. Precipitation was allowed to proceed for 30 min or more during which time the tubes were kept in an ice bath. The small amounts of precipitated material were then collected on either 8.0- or 0.8-μ Millipore filters in the cold. The filters were washed with Krebs solution containing the same concentration of ammonium sulfate, air dried and stored at freezer temperatures.

The precipitate was removed by intensive maceration and shaking of the filter with cold Krebs solution containing 1 mg reduced glutathione in each milliliter. The solutions were kept in an ice bath at all times. Samples were taken for protein analysis.

Protein was determined by the method of Lowry et al. (3), with bovine serum albumin as the standard and a blank containing glutathione when necessary. The absorbance at 250 μm, determined after deproteinizing with 5% perchloric acid, was taken as an estimate of purine and pyrimidine derivatives. The results are expressed as “adenylates” because 5-adenylic acid (Calbiochem) was used as the standard. The inotropic activity of the protein removed from the Millipore filters was estimated on isolated left atria from kitten hearts. These were suspended in Krebs-Henseleit solution containing 11 mM glucose and contractile failure was induced by prolonged (2 to 4 hr) electrical stimulation at rates of 4 to 5 beat/sec. The substance was tested when the atria had failed so that their contractile force was 30% of the initial force, as determined during stimulation at a rate of 1/sec.

Ouabain and digitoxin were obtained from Nutritional Biochemicals Corp. and were used without further purification. Dihydrodigitoxin was synthesized as described by Jacobs and Hoffman (4) by catalytic hydrogenation of digitoxin which had been recrystallized once from methanol-water. The product was recrystallized repeatedly from methanol-water. Infrared analysis indicated that it contained 85 to 95% dihydrodigitoxin.

Results

EFFECTS OF OUABAIN ON CONTRACTILE FORCE OF GAS-PERFUSED HEARTS

Our earlier report indicated that gas-perfused hearts responded normally to most drugs, including sympathomimetic amines and their blocking agents, and cholinergic drugs (1). An outstanding exception to this was the lack of response to ouabain. When this agent at concentrations of $1 \times 10^{-9}$ to $1 \times 10^{-7}$ g/ml was added to the Krebs solution preperfusing the heart prior to gas perfusion, it did not affect the strength of contraction or the time course of the slow decline in contractile force of gas-perfused hearts. These concentrations of ouabain had no positive inotropic effect when 5 ml of Krebs solution containing the drug was injected into hearts after 4 to 7 hr of gas perfusion, at which time contractile force had decreased to 25 to 70% of control. This treatment was also ineffective in reversing washout cardiac failure (see next section). However, preperfusion with Krebs solution containing a digitalis glycoside resulted in a considerable decrease in the incidence of contractile alternans among other strengths of contraction usually in the absence of changes in electrical activity) which we had described previously (1). This effect is summarized as part of Table 1.

THE EFFECT OF INTERMITTENT PERFUSION ON THE CONTRACTILE FORCE OF GAS-PERFUSED HEARTS

Hearts, electrically driven at 168 beat/min, were perfused with gas for 1 hr. Five milliliters of substrate-free Krebs solution was then injected every 30 min until a total of 20 ml had been given. Each of these injections will hereafter be referred to as a “wash,” the liquid collected after passage through the heart as a “washing,” and the effect of a series of four washes as “washout failure.”

The immediate effect of each wash was a transient (1 to 5 min) doubling of contractile force. This was followed by either an immediate or a gradual fall in contractile force to below that obtaining before the wash. Figure 1 shows one such response of a heart in which there was a rapid decrease in contractile force. The decreases in contractile force...
DIGITALIS AND WASHOUT CARDIAC FAILURE

TABLE 1

Effects of Digitalis Glycosides on Washout Failure, Total Protein and Adenylate Content of Washings, and Incidence of Contractile Alternation

<table>
<thead>
<tr>
<th></th>
<th>Percent contractile force after washout</th>
<th>Total protein in washings*</th>
<th>Total adenylate in washings*</th>
<th>No. of hearts with contractile alternation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not pretreated</td>
<td>34 ± 6%</td>
<td>664 ± 55</td>
<td>140 ± 10</td>
<td>3/4</td>
</tr>
<tr>
<td>Ouabain 1 × 10^-10 g/ml</td>
<td>44 ± 5%</td>
<td>563 ± 28†</td>
<td>154 ± 17†</td>
<td>2/4</td>
</tr>
<tr>
<td>Dihydrodigitoxin 1 × 10^-8 g/ml</td>
<td>58 ± 2%</td>
<td>512 ± 9</td>
<td>158 ± 12†</td>
<td>3/8</td>
</tr>
<tr>
<td>Ouabain 3 × 10^-10 g/ml</td>
<td>61 ± 4%</td>
<td>486 ± 18</td>
<td>142 ± 14†</td>
<td>0/4</td>
</tr>
<tr>
<td>Ouabain 1 × 10^-6 g/ml</td>
<td>81 ± 4%</td>
<td>378 ± 18</td>
<td>147 ± 17†</td>
<td>0/4</td>
</tr>
<tr>
<td>Digoxin 1 × 10^-6 g/ml</td>
<td>88 ± 5%</td>
<td>369 ± 154†</td>
<td>149 ± 11†</td>
<td>0/6</td>
</tr>
<tr>
<td>Not washed</td>
<td>85 ± 6%</td>
<td></td>
<td></td>
<td>4/4</td>
</tr>
</tbody>
</table>

Total perfusion time 3 hr after 5 min Krebs preperfusion. Intermittent injection of 5 ml Krebs solution at 60, 90, 120 and 150 min. Digitalis glycosides added to preperfusion fluid.

*As micrograms per gram wet wt., means ± standard error.
†Not significantly different from untreated group (P > 0.05).
‡Not significantly different from ouabain 1 × 10^-9 g/ml group (P > 0.05).

FIGURE 1

Effect of a wash with Krebs solution on the isometric contractile force of a gas-perfused heart. Resting tension maintained at 10 g. Heart rate = 168/min. Extreme right: contractile force 10 min after wash.

were cumulative during the washout procedure. The bottom curve in Figure 2 shows the time course of washout failure in a group of 4 untreated hearts. Contractile force 30 min after the last wash (3-hr perfusion), expressed as a percentage of that measured at the beginning of gas perfusion, was 34 ± 6%. Without washing, contractile force after 3 hr of perfusion was 85 ± 6%.

Ouabain, 1 × 10^-10 to 1 × 10^-6 g/ml, was added to the Krebs solution used to preperfuse groups of 4 hearts. These hearts were next perfused with gas for 60 min and were washed four times. The washes did not contain ouabain. Figure 2 shows that these concentrations of the glycoside provided dose-related protection against washout failure. The highest concentration protected completely.

The maximally effective concentration of ouabain in our experiments was 10 to 100 times less than is usually needed to obtain a measurable inotropic effect on isolated cardiac tissue. It appeared possible that protection from washout failure was due to an action different from that causing an increase in contractile force. We therefore compared the activities of digoxin and of dihydrodigitoxin, compounds which differ very little in structure or surface activity but whose positive inotropic activities differ by a factor of 10 to 25 in the usual tests (4). Table 1 shows that digoxin, 1 × 10^-9 g/ml, had an effect equal to that of the same concentration of
The effect of intermittent perfusion with Krebs solution on the contractility of gas-perfused hearts and prevention of this effect by graded concentrations of ouabain added to the preperfusion fluid. Top curve: contractile force of control hearts. Bottom curve: contractile force of hearts given 5 ml of Krebs solution at W. The other curves show the effects of ouabain at the concentrations indicated. Four hearts were used in each group. Vertical bars indicate ±1 SE.

The effect of dihydrodigitoxin, $1 \times 10^{-8}$ g/ml, was approximately equivalent to the effect of ouabain, $3 \times 10^{-10}$ g/ml. These results yield a potency ratio between ouabain or digitoxin and dihydrodigitoxin of 20 to 30.

A sample of each washing was analyzed for protein and for "adenylates." Table 2 shows that both the protein and adenylate contents of the first washings were high relative to those of subsequent washings. Most washings from hearts pretreated with effective concentrations of ouabain contained less protein than the corresponding ones from control hearts. There was no difference in the content of "adenylates" removed from control and pretreated preparations. Differences in protein content between sequential washes (Table 2) were greater after preperfusion with ouabain.

Table 1 shows the total amounts of protein and "adenylates" removed from hearts under the conditions already described. It is clear that protection from washout failure by the digitalis glycosides was accompanied by a decrease in the protein, but not the adenylic acid derivative, content of the washings.

<table>
<thead>
<tr>
<th>Protein and Adenylate Contents of Washings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash</td>
</tr>
<tr>
<td>------</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>Adenylates in Washings</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
</tbody>
</table>

*As micrograms per gram wet wt., means ± standard error.
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TABLE 3

Responses of Isolated Atria to Cardioactive Material

<table>
<thead>
<tr>
<th>Ouabain preperfusion (g/ml)</th>
<th>None</th>
<th>$1 \times 10^{-9}$</th>
<th>$3 \times 10^{-10}$</th>
<th>$1 \times 10^{-10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Mean protein in bath (µg/ml)</td>
<td>12.5</td>
<td>10.6</td>
<td>9.0</td>
<td>6.8</td>
</tr>
<tr>
<td>Range</td>
<td>7-21</td>
<td>4-18</td>
<td>5-16</td>
<td>4-11</td>
</tr>
<tr>
<td>Incidence of samples with scores*</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>8</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>3</td>
<td>5</td>
<td>13</td>
</tr>
<tr>
<td>B. Mean of scores</td>
<td>2.5</td>
<td>1.31</td>
<td>1.08</td>
<td>0.43</td>
</tr>
<tr>
<td>&quot;Specific activity&quot; (B/A)</td>
<td>0.200</td>
<td>0.124</td>
<td>0.118</td>
<td>0.063</td>
</tr>
</tbody>
</table>

*Scoring: All atria had failed to 25 to 30% of their initial force. 0 = no effect; 1 = increase in force to < 40% of initial; 2 = increase in force to 40 to 50% of initial; 3 = increase in force to > 50% of initial.

CONTRACTILE FORCE

Effect of the precipitate from washings of gas-perfused hearts on the contractile force of a failed kitten atrium. At A, material added to bath (48 µg/ml as protein).

The amounts of material added to the bath varied considerably, the final concentration ranging from 4 to 21 µg/ml (mean of 9.7 µg/ml). Table 3 indicates two effects of ouabain. There was a decrease in the amount of material precipitated by 25% saturation with ammonium sulfate as mirrored by the final concentration of protein in the bath. In addition, the material from treated hearts had a lower "specific activity," i.e., there was a smaller increase in contractile force per microgram of protein. Control experiments showed that small amounts of ammonium sulfate dried on Millipore filters or of glutathione alone added to the bath had no positive inotropic effect. The maximal response obtained from the material in the assay system was a
return to 75 to 85% of the contractile force measured before failure was induced by driving. One such maximal response, obtained at a protein concentration of 48 \( \mu g/ml \), is shown in Figure 3.

The biological activity of the material was found to be highly unstable. Storage of the dissolved material at room temperature resulted in partial or complete inactivation within 45 min. Glutathione, 1 mg/ml, slowed this inactivation, especially if the solution was stored in the cold.

Discussion

Perfusion of isolated hearts with a saline medium invariably leads to failure of the preparation. Addition of substrates such as glucose or pyruvate delays this process but cannot prevent it. Addition of serum causes considerable further delay in failure, an observation first made by Bowditch (5) and confirmed many times since (2, 6, 7). There have been a number of attempts to isolate and identify the active serum component. Hajdu et al. (8) considered \( \beta \)-palmitoyllysolecithin from serum important for contractility in frog hearts but this substance appears to have little effect in mammalian preparations (9). They have recently isolated a complex protein system, the cardioglobulins, which also has a positive inotropic effect on frog hearts (10, 11). Kinekard, a large polypeptide possessing inotropic activity, has been obtained from plasma by Nayler et al. (12). Zachariah (13) has shown that a lipid extract of plasma is as effective on rat hearts as is whole plasma.

Viability of the isolated heart may not only depend on substances reaching it through the circulation but may also be dependent on endogenous materials. Clark and coworkers (2) observed that recirculation of the fluid perfusing frog and mammalian hearts prolonged the useful life of the preparations. They suggested that loss of a lipoid material was responsible for failure and showed that addition of impure, but not of purified, lecithin increased contractile force. Relatively little has been done to exploit their observations. We believe that the present results strongly implicate the loss of an inotropic material into the perfusion fluid as a cause for failure in Langendorff preparations. Prevention of this loss by perfusion with a gas mixture instead of a liquid would explain the long survival of gas-perfused hearts which we described earlier (1) and of hearts perfused with fluorinated hydrocarbons (14).

We have demonstrated the existence of a cardioactive material which can be removed from gas-perfused hearts by short periods of perfusion with Krebs solution. The degree of failure caused by intermittent flushing with a total of only 20 ml of liquid is equivalent to that seen during the same period (2 hr) of continuous perfusion with the same medium (1). We have shown failure to be correlated roughly with loss of protein from the hearts and prevention of failure by digitalis glycosides to be accompanied by a decrease in the washout of protein, but not of "adenylates" from the isolated hearts. It cannot be taken for granted, however, that the material showing activity is a protein. Pretreatment with digitalis decreased the biological activity of the precipitated material more than its protein content. This indicates that the active substance is either one of several proteins in the precipitate or a nonprotein material coprecipitated under our conditions. We used the isolated kitten atrium failed by prolonged driving for the preliminary bioassay of the material because we found that it was more responsive than "normal" preparations. The greater activity in failed atria is consistent with the hypothesis that the material replaces a substance lost from failed myocardium.

The instability of the material, the fact that it may be precipitated with ammonium sulfate and that it is released from contracting muscle, suggests that we may be dealing with a factor similar to the muscle activity factors isolated and described by Havivi and Wertheimer (15) and by Goldstein (16). This similarity is emphasized by our preliminary observations that the material may increase the transport of some nonutilizable sugars into skeletal muscle.

Our data suggest the possibility that digi-
Digitalis and Washout Cardiac Failure

talis glycosides may exert their therapeutic effect by slowing or halting the loss of an intrinsic inotropic substance from failing hearts. The concentrations necessary for this effect are lower than those usually observed to have an inotropic action. They are within the range for which activation of Na-K-activated ATPase has been reported (17) and are considerably below the concentrations necessary to inhibit this enzyme. It should be pointed out, however, that we have no evidence that the material is lost in vivo or that the present observations account for the usual therapeutic effect of digitalis.

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References

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