RELEASE OF CATECHOLAMINE BY OUABAIN

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ABSTRACT

The purpose of this study was to determine whether a cardiac glycoside could release catecholamine from sympathetically innervated tissues. Isolated cat spleen strips were used because they provide a preparation with a high noradrenaline content and smooth muscle which contracts when activated by adrenergic drugs. Ouabain caused a marked contraction. The data indicated that the contraction was due to noradrenaline release. The contraction was nearly abolished by the α adrenergic blocking agent phenoxybenzamine. Ouabain failed to cause contractions of strips from spleens depleted of noradrenaline either by chronic denervation or treatment of the cats with reserpine. Moreover, ouabain protected noradrenaline receptors from blockade by phenoxybenzamine in strips which had not been depleted of noradrenaline, but failed to protect in strips from cats treated with reserpine.

Several observations suggested that the release of noradrenaline by ouabain was due to downhill ion movement, i.e. loss of cellular potassium and gain of sodium. There was a long latent period between the addition of ouabain to the bath and the onset of contraction, and the length of this period was inversely related to concentration. This finding parallels the observations of others who studied downhill ion movement caused by cardiac glycosides. The β adrenergic blocking agent pronethalol opposed both the downhill ion movement and the release of noradrenaline induced by ouabain. The release of noradrenaline by ouabain

was antagonized by a high calcium concentration in the bathing fluid; this is known to stabilize the membrane of excitable cells and thereby cause it to resist ion movements. Also, when the amount of ion available to run downhill was reduced by substitution of sucrose for sodium chloride in the bathing fluid, noradrenaline release was inhibited. Replacing the sodium chloride of the bathing medium with potassium chloride prevented contractions caused by ouabain, but not those caused by exogenous noradrenaline. High extracellular potassium is known to oppose the effect of cardiac glycosides on ion movements.

In dogs, depletion of catecholamines by treatment with reserpine did not impair the ability of pronethalol to convert ouabain-induced arrhythmias to sinus rhythm. This indicated that neither prevention of catecholamine release nor blockade of catecholamines at their receptor sites in the heart is responsible for the antiarrhythmic effect. Infusion of pronethalol in an antiarrhythmic dose in dogs treated with reserpine, but without ouabain-induced arrhythmias, increased the normal sinus rate and the rate of firing of subatrial pacemakers elicited by vagal stimulation. This is inconsistent with a generalized cardiac depression as the mechanism of pronethalol's antiarrhythmic effect.

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INTRODUCTION

PART I

The idea that catecholamines can be released by digitalis (see footnote) originated with the work of Tanz (1960). He found that dichloroisoproterenol (DCI) prevented the positive inotropic effect of ouabain on the isolated cat papillary muscle, and that the effect was absent in papillary muscles from cats pretreated with reserpine. Since it was known that DCI blocked the inotropic action of catecholamines (Moran and Perkins, 1958) and that reserpine depleted the heart of catecholamines (Paasonen and Krayer, 1958) Tanz concluded that "one of the mechanisms whereby ouabain increases contractile force is by the release of catecholamines".

The idea was extended by Cairoli, Reilly, and Roberts (1961) who also found that the inotropic effect of ouabain on cat papillary muscles was reduced if the cats were pretreated with reserpine. They also showed that the effect was not due to an unspecific depressant effect of reserpine, because muscles from cats pretreated with reserpine responded to adrenaline as strongly as muscles from untreated cats. Cairoli et al., noticed that ouabain usually caused spontaneous beating in papillary muscles from untreated cats, but not in muscles taken from cats pretreated with reserpine. As a result of this observation they suggested that the ability of ouabain to induce spontaneous beating was due to catecholamine release.

Throughout the thesis the word digitalis is used as a general term to mean one or all of the active cardiac glycosides. It is generally assumed that the actions of different cardiac glycosides are essentially the same, and that they differ only in their onset and duration of action and potency. It is apparent from the variety of glycosides used by different investigators that this assumption is accepted by most. Whether this has contributed to the controversy concerning release of catecholamines by digitalis is not certain.

Yelnosky and Ervin (1961) were the first to investigate the role of catecholamines in digitalis-induced arrhythmias and augmentation of contractile force *in vivo*. By giving cumulative doses of ouabain to anesthetized dogs, they studied changes in contractile force with the Walton-Brodie (Boniface, Brodie and Walton, 1953) strain gauge arch and assessed changes in rhythm from the ECG. In contrast to Tanz and Cairoli *et al.*, they found that depletion of catecholamines by pretreatment with reserpine or blockade of catecholamines by DCI failed to alter the effect of ouabain on the heart.

In an attempt to resolve the discrepancy Roberts and co-workers (1963) carried out an extensive study on three different preparations: cat papillary muscle, the dog with surgically-induced heart block, and the intact cat. The results from the papillary muscles showed that the positive inotropic effect of ouabain was not diminished by pretreatment of the cats with reserpine. However, in agreement with Cairoli *et al.*, the incidence of ouabain-induced spontaneous beats was significantly reduced in these muscles.

In the dog, the effect of acetylstrophanthidin on ventricular automaticity was examined before and after giving βTM 10; this compound, like TM 10, blocks the release of catecholamines from adrenergic nerves (Exley, 1957; McLean, Geus, Pasternack, Mattis and Ullyot, 1960). Atrial influence was excluded by ligating the bundle of His so that ventricular rate could be used as an index of ventricular automaticity. The authors concluded that catecholamine release contributed to the acetylstrophanthidin-induced increase in ventricular automaticity because the acceleration in ventricular rate caused by strophanthidin was significantly less after βTM 10. Unfortunately, they made no attempt to demonstrate whether two successive challenges with acetylstrophanthidin in the absence of

βTM 10 caused equal changes in ventricular rate. They also failed to show whether the only action of βTM 10 which could account for the observed effect was the inhibition of catecholamine release.

In their experiments on intact cats a modification of the "vagus amine test" (Roberts, Standaert, Kim and Riker, 1956) was used. With this method the sinus rate is slowed by vagal stimulation to a point just short of that where either the A-V node or ventricle becomes the pacemaker. Under these conditions ectopic beats are easily induced by agents which raise ventricular automaticity, e.g. sympathomimetic amines or cardiac glycosides. They found that pretreatment with reserpine significantly increased the dose of acetylstrophanthidin necessary to cause ectopic beats, and the sensitivity of the heart was restored by replenishing catecholamine stores with infusions of noradrenaline.

In contrast to the small doses of strophanthidin which induced arrhythmia in combination with vagal stimulation, large doses were required to cause arrhythmia in the absence of vagal stimulation, and in this case pretreatment with reserpine had no effect. From these experiments the authors concluded that catecholamine release is the primary mechanism involved in the arrhythmia produced by small doses of digitalis, but that another mechanism also plays a role in arrhythmias caused by large doses. Moreover, they pointed out that their large doses of acetylstrophanthidin were comparable to the doses of ouabain used by Yelnosky and Ervin, and suggested that this explained the negative results of that study.

The experiments of Roberts et al., are open to question. It is likely that vagal stimulation caused a hypotensive response which initiated a reflex sympathetic discharge. This reflex may have been respon-

sible for the release of catecholamines, which appeared to contribute to the increased ventricular automaticity seen after acetylstrophanthidin, rather than a direct releasing action of the glycoside on the cardiac adrenergic nerves.

In the same year as Roberts et al., Morrow, Gaffney and Braunwald (1963) published results which militated against catecholamine involvement. They found in dogs that after almost complete depletion of cardiac catecholamines by either reserpine pretreatment or chronic sympathetic denervation the inotropic effect of ouabain was unchanged. Similarly, neither treatment altered the dose of ouabain required to produce ventricular premature contractions. Unlike the data of Yelnosky and Ervin, these data cannot be reconciled with those of Roberts et al. on the basis of glycoside dosage, for the doses of ouabain used in this study were comparable to those used by Roberts et al.

Yet other reports in favor of the catecholamine hypothesis appeared. Erlij and Mendez (1963, 1964) showed that reduction of adrenergic influence on the heart modified the lethal effects of digitoxin. They used three different procedures: pretreatment with reserpine, treatment with the β adrenergic blocking agent pronethalol, and acute removal of the thoracic chain and adrenals. Intoxication with digitoxin after any one of these procedures caused cardiac arrest, whereas in control animals it caused ventricular fibrillation. Moreover, if the arrested hearts were given noradrenaline they fibrillated. Erlij and Mendez concluded from these observations that digitoxin's action on cardiac automaticity depended on the presence of catecholamines. A more recent report (Takagi, Zanuttini, Khalil and Bellet, 1965) showed that the lethal dose of digi-

toxin was higher in dogs pretreated with reserpine than it was in control dogs, but the authors noted that all the dogs died of ventricular fibrillation regardless of reserpine pretreatment.

In view of so much controversy Tanz (1964) re-evaluated the effects of reserpine pretreatment and DCI on the inotropic effect of ouabain in the cat papillary muscle. The results were essentially the same as those first reported (1960). However, in the more recent study it was shown that papillary muscles from cats pretreated with reserpine, or papillary muscles treated with DCI responded as strongly to an increased calcium concentration as the control muscles. This indicated that the treated muscles were not unspecifically depressed. The author concluded that there were two possible mechanisms which could explain the apparent relationship between the inotropic effect of ouabain and the availability of endogenous catecholamines in the heart. The first was that ouabain may act to increase the contractile force by releasing catecholamines from storage sites located within the heart. The second was that the inotropic effect depended upon the presence of a certain level of catecholamines which would be analogous to the permissive action of adrenal cortical hormones.

Tanz supported his work on papillary muscles by studies on Langendorff preparations made from cats pretreated with either reserpine or guanethidine (Tanz and Marcus, 1966). The cardiac catecholamine content, compared with those of untreated cats, was reduced to less than two per cent by reserpine and to eight per cent by guanethidine. Both drugs caused a significant decrease in the inotropic response to ouabain.

Cardiac effects of reserpine other than catecholamine depletion.

A great deal of work thus far cited has relied on reserpine pretreatment. In many cases where this treatment modified the effect of a cardiac glycoside it was taken as evidence that the glycoside had, as one of its actions, the ability to release endogenous catecholamines. Although the most outstanding pharmacological action of reserpine is depletion of catecholamines, this does not rule out the possibility that other actions might account for the observed changes in the effects of glycosides after reserpine.

Several reports provide experimental support for this objection. Boyajy and Nash(1963) showed that the Rauwolfia alkaloid ajmaline, which does not deplete tissues of catecholamines, protected dogs against ouabain toxicity as effectively as reserpine did. Spann and co-workers (1965) showed that the inotropic effect of strophanthidin was as great in papillary muscles from cat hearts depleted of catecholamines by chronic cardiac denervation as it was in papillary muscles from normal hearts. However, they found that the inotropic effect was significantly reduced in papillary muscles from cats previously poisoned with exceedingly large doses of reserpine (3.0 mg/kg/day for 2 days). Innes and Krayer (1958) found that reserpine had a depressant effect on the heart which was independent of catecholamine depletion. When reserpine was injected acutely into heart-lung preparations made from dogs previously depleted of catecholamines by chronic treatment with reserpine it produced a negative chronotropic and inotropic effect.

Withrington and Zaimis (1961) reported that twenty-four hour pretreatment of the cat with reserpine (1.0 mg/kg) caused heart failure.

Their conclusions were based on the appearance of the heart and contractile force measurements made with a strain gauge arch. They found that the heart was enlarged and the venae cavae and their tributaries were engorged. The contractile force in reserpine pretreated animals was one-third to one-half that of untreated animals.

Withrington and Zaimis noted that the heart failure caused by reserpine was similar to that caused by hypoxia, and suggested the possibility of reserpine causing a defect in myocardial metabolism. Levy and Richards (1965) tried to correlate an altered inotropic response with an altered oxidative metabolism. They measured the increased force of contraction and oxygen consumption caused by ouabain in atria isolated from rabbits pretreated with reserpine and from untreated rabbits. With reserpine the inotropic response to ouabain was significantly less, but the ouabain-induced increase in oxygen consumption was unchanged. They also noted, in contrast to Withrington and Zaimis, that the initial contractility (before ouabain was added) was the same in both groups of atria. In view of their negative results the authors concluded that an impaired oxidative metabolism failed to explain the effect of reserpine on the inotropic action of ouabain.

PART II

THE EFFECT OF β ADRENERGIC BLOCKING AGENTS ON DIGITALIS-INDUCED ARRHYTHMIAS.

A β adrenergic blocking agent can be broadly defined as a drug which blocks the smooth muscle inhibitory and cardiac excitatory actions of catecholamines. Until 1962 the only compound known to have these properties was dichloroisoproterenol (DCI) (Powell and Slater, 1958; Moran and Perkins, 1958; Dresel, 1960), but several agents are now available (Black and Stephenson, 1962; Shanks, 1965; Folle and Aviado, 1965; Stanton, Kirchgessner and Parmenter, 1965; Kvam, Riggilo and Lish, 1965; Somani and Lum, 1965). The most fully studied of these is pronethalol (Dornhorst and Robinson, 1962; Sekiya and Vaughan Williams, 1963; Donald, Kvale and Shepherd, 1964; Chamberlain and Howard, 1964; Koch-Weser, 1964).

Several instances have already been cited where β adrenergic blocking agents were used to determine whether catecholamines were involved in the cardiac effects of digitalis. In these studies there was little agreement among the various authors as to the effect. For example, Tanz (1960, 1964, 1966) reported that DCI reduced the inotropic response to ouabain, and Erlij and Mendez (1963, 1964) showed that the toxic effects of digitoxin were modified by DCI; but Yelnosky and Ervin (1961) found that DCI failed to alter the inotropic action of ouabain. In contrast to this controversy there is general agreement that either DCI or pronethalol given during an already established digitalis-induced cardiac arrhythmia has a salutary effect (Lucchesi and Hardman, 1961; Lucchesi, 1964, 1965; Somani and Lum, 1965; Tuttle and Innes, 1964, 1966).

For the most part the authors who have employed β adrenergic

blocking agents to treat digitalis-induced arrhythmias have not invoked blockade of endogenous catecholamines to explain their results. The first report that Vaughan Williams and Sekiya (1963) made, however, was an exception. They found in guinea-pigs that either DCI or pronethalol antagonized the ventricular fibrillation resulting from ouabain intoxication, and with no further evidence concluded that the effect was due to blockade of "β sympathetic actions". Apparently they were unaware of an earlier work by Lucchesi and Hardman (1961) which indicated that the ability of DCI to oppose arrhythmias caused by digitalis was independent of β adrenergic blockade. This work showed that DCI reversed digitalis-induced arrhythmias in isolated rabbit heart and in the intact dog. However, the protection afforded by DCI in the rabbit heart did not persist and could be demonstrated only when high concentrations of DCI were present in the perfusion fluid, but β adrenergic blockade did persist and was still present (as judged by inhibition of the chronotropic action of adrenaline and isoproterenol) after DCI was removed from the perfusion fluid. This study also showed that compounds chemically related to DCI, but devoid of β adrenergic blocking activity, were as effective as DCI against the arrhythmias in the rabbit heart and intact dog.

Lucchesi (1964, 1965) later carried out similar studies with pronethalol on the same two preparations. As with DCI he found that pronethalol antagonized acetylstrophanthidin-induced arrhythmias in the isolated rabbit heart when it was present in the perfusing medium, but after pronethalol was removed from the medium the arrhythmia reappeared even though β adrenergic blockade was still present. Moreover he showed that the dose of pronethalol needed to reverse acetylstrophanthidin- or ouabain-induced arrhythmias in the intact dog greatly exceeded that neces-

sary to block β receptors. Further evidence that β adrenergic blockade was unnecessary for the antidigitalis activity was provided by experiments showing that the dextro isomer of pronethalol was as effective as the racemic mixture against the arrhythmias even though it was much less potent as a β adrenergic blocking agent (Lucchesi, 1965). Still more evidence was supplied by Somani and Lum (1965) who showed that N-isopropyl-p-nitrophenylethanolamine (INPEA) was a potent β adrenergic blocker but had no effect on ouabain-induced arrhythmias in dogs.

Since the ability of DCI and pronethalol to antagonize digitalis-induced arrhythmias could not be explained by β adrenergic blockade, Somani and Lum suggested that the antiarrhythmic effect was due to an unspecific "quinidine-like" action. This suggestion was supported by an earlier work of Sekiya and Vaughan Williams (1963), who postulated that interference with depolarization was the essential feature of "antifibrillatory" drugs. They found in isolated rabbit atria that both pronethalol and quinidine slowed the rate of rise of the action potential and reduced its overshoot.

PART III

THE SPLEEN STRIP.

Part I pointed out how little agreement there is on whether cardiac glycosides promote the release of catecholamines. The controversy was almost inevitable since the heart was invariably the experimental object. Even the strongest advocates of catecholamine release acknowledged that more was involved in digitalis-induced changes in cardiac rhythm and contractility. Because of this, any data suggesting that catecholamine release was contributing to either changes in rhythm or contractility were unavoidably complicated.

However, there is no reason why all studies involving digitalis must be done on the heart. With the exception of the adrenal medulla the chief source of catecholamines is the sympathetic postganglionic nerve fibres. Then, if a drug causes release of catecholamines from an organ other than the medulla, it must do so through an effect on these fibres, and it is not unreasonable to assume that the response of these fibres to a drug is similar regardless of their location. Therefore, if digitalis releases catecholamines from the heart it should release them from any organ with sympathetic innervation. This hypothesis initiated the research described in this thesis.

The isolated cat spleen strip was chosen because it provided an *in vitro* preparation with a rich sympathetic innervation (von Euler, 1956) and smooth muscle known to be responsive to noradrenaline. Most of the catecholamine present in the spleen is noradrenaline (von Euler, 1956), and for this reason the term noradrenaline release is used rather than catecholamine release throughout the thesis. Ouabain was chosen

over other fast acting cardiac glycosides because it was the one most often used by other workers, and it was one with which I had some previous experience (Tuttle and Innes, 1964, 1966).

The isolated spleen has been little used for pharmacological studies. It was first described by Sherrington in 1919 who showed that adrenal extracts caused contraction of rabbit spleen strips. Ten years later similar observations were made by Fredericq (1929) with isolated strips from dog spleen. In 1933 Vairel showed that adrenaline contracted spleen strips from dog, rabbit, frog, and tench. Saad (1935) extended these observations to man, cat, guinea-pig, rat, and buffalo, and reported that the contractions were blocked by ergotoxine. In 1962 Bickerton, Rockhold, and Micalizzi used cat spleen strips to assay adrenergic blocking agents, and Innes showed that 5-hydroxytryptamine and adrenaline acted on the same receptors in the cat spleen strip. The most recent paper on this preparation was by Bickerton (1963) who showed that the contractions caused by adrenaline, noradrenaline, and isoproterenol were due to activation of the α-adrenergic receptors as classified by Ahlquist (1948).

PART IV

THE EFFECT OF DIGITALIS ON IONIC BALANCE.

Experiments which will be described in the results showed that ouabain could release noradrenaline, and an attempt was made to determine whether this was related to ouabain's effect on ionic balance. The first observations suggesting that digitalis had an influence on ionic balance were made by Harrison, Pelcher, and Ewing (1930). They found that the hearts of patients who had been treated with digitalis contained less potassium at autopsy than the hearts of other patients. This finding stimulated Calhoun and Harrison (1931) to measure the myocardial concentration of potassium in dogs given digitalis. The dogs were divided into a control group and three experimental groups designated "therapeutic", "toxic", and "fatal"; these designations reflected the doses of digitalis given. There was a significant lowering of potassium in the "toxic" and "fatal" groups, but the potassium concentration of the "therapeutic" group was only three per cent below that of controls. They concluded that while toxic doses of digitalis lowered myocardial potassium the effect of therapeutic doses was at most very slight. Since then a vast number of studies have been done to determine the effect of digitalis on myocardial potassium, and it is now generally agreed that toxic doses promote the loss of cellular potassium (Conn and Luchi, 1964). However, controversy still exists as to whether therapeutic doses cause a loss of myocardial potassium (Luchi and Conn, 1965).

Any studies involving the heart are unavoidably complicated by changes in rhythm and contractility, and for this reason many investigators have considered the red blood cell as an ideal preparation for studying the ionic effects of digitalis. Not only does it circumvent the pro-

blems of rhythm and contractility, it has a further advantage in that there is no extracellular space to consider. Like most animal cells the red cell has a high intracellular ratio of potassium to sodium, but when the cells are stored in the cold there is a "downhill" movement of sodium and potassium, i.e. the potassium/sodium ratio decreases and approaches that of the bathing medium. However, on rewarming the original ratio is restored, and since this requires an "uphill" accumulation of potassium and extrusion of sodium against a concentration gradient the process is considered to be active (Kahn, 1963; Glynn, 1964).

In 1953, Schatzmann showed that strophanthidin K prevented the uphill movement of sodium and potassium when cold-stored red cells were rewarmed. He concluded that the inhibition could not be explained by interference with cell metabolism because strophanthidin had no effect on oxygen consumption or lactic acid production. Nor could it be explained by an increase in passive permeability of the cell membrane because strophanthidin failed to accelerate the downhill ion movement that took place in the cold. Therefore it appeared that strophanthidin directly inhibited the active extrusion of sodium and accumulation of potassium. This was soon confirmed by Joyce and Weatherall (1955) who showed that digitoxin and other cardiac glycosides inhibited the uptake of K^{42} by red cells. There have since been many studies on the effects of cardiac glycosides on potassium and sodium movements in red cells which support this idea (for reviews see Glynn, 1964; Judah and Ahmed, 1964; Skou, 1965).

The ability of cardiac glycosides to inhibit uphill cation movement is not limited to the red cell. Wherever active transport of sodium and potassium has been shown to take place, it has been possible to depress it with cardiac glycosides, e.g. cardiac muscle (many species),

various smooth muscle (many species), nerve (squid), erythrocyte (man, pig, lamb), skin (frog), kidney (dog), lens (cow), tumour cells (mouse), larvae (beetle), whole animal (planaria), and thyroid (sheep) (Khan, 1963; Glynn, 1964).

The essential factor involved in this inhibition has recently been elucidated. It is known that energy derived from breakdown of ATP is necessary for the uphill transport, and at least part of the ATP breakdown is due to the action of an enzyme located in the cell membrane which can be inhibited by cardiac glycosides (Caldwell, Hodgkin, Keynes and Shaw, 1960; Dunham and Glynn, 1961; Glynn, 1962). This enzyme has been named "transport" ATPase (Glynn, 1964). There are several reasons for thinking that inhibition of this enzyme explains the action of cardiac glycosides on active cation transport: Sodium and potassium must be present for activation of the enzyme (Glynn, 1962). The concentrations of glycosides necessary to inhibit the enzyme from any particular tissue are the same as those required to inhibit transport (Dunham and Glynn, 1961). Structural modifications in the glycoside molecule cause similar changes in potency for both enzyme and transport inhibition (Repke, 1963). The degree of inhibition for both transport and enzyme activity is dependent on the time that the glycoside is allowed to act (Glynn, 1957, 1964). An increase in extracellular potassium concentration decreases the effect of the glycosides on both transport and enzyme inhibition (Dunham and Glynn, 1961).

The general picture which has emerged is that the steady-state concentrations of ions, i.e. the high intracellular ratio of potassium/sodium is the result of a balance between active uphill transport (which depends on ATP breakdown by ATPase) and passive downhill movement along

the concentration gradient. Therefore cardiac glycosides upset this balance and lead to downhill movement which results in an increased intracellular sodium and decreased intracellular potassium concentration. Kahn (1963) has pointed out that the active accumulation of potassium and extrusion of sodium are linked, and that it has not been possible to affect one without affecting the other.

METHODS

General procedure for experiments on spleen strips.

Young cats of either sex weighing between 0.3 and 2.0 kg were killed by a blow on the head. The spleen was immediately removed and immersed in Krebs-Henseleit solution at room temperature. After adhering tissue and blood were removed, the spleen was placed on moist filter paper and one to four strips 25 mm long and 2 to 3 mm wide were cut from the edge. Each strip was suspended in an organ bath containing 15 ml of Krebs-Henseleit solution kept at 38°C and bubbled with 95% oxygen and 5% carbon dioxide. Isotonic contractions at one gram tension were amplified six fold and recorded on a kymograph.

The strips were suspended for sixty minutes before drugs were added. The bathing fluid was changed every fifteen minutes except in tests with ouabain which required long exposure. Unless otherwise specified, drugs which caused contraction were left in the bath until the contraction reached maximum. Exposure time to phenoxybenzamine and pronethalol varied and is specified in the results.

Chronic denervation of the spleen.

The cats were sedated with an intraperitoneal injection of pentobarbital sodium (17 mg/kg), and then anesthetized with ether by the open drop method. The spleen was exposed through a midline abdominal incision. Denervation was done by stripping the splenic arteries of periarterial nerves and removing the loose connective tissue between the splenic arteries. The spleen was then replaced in the abdomen and the incision sutured. A 1.0 ml mixture of 400,000 units of penicillin G and 0.5 g streptomycin (Fortimycin, Ayerst) was injected intramuscularly as prophylaxis against infection. Fourteen days after denervation the cats

were killed and spleen strips were prepared in the usual way.

Potassium assay.

After tests of their activity in the organ bath the strips were removed, blotted on dry filter paper, and weighed on an analytical balance. Each strip was then homogenized with a motor driven Teflon pestle in 1.0 ml of 10% trichloroacetic acid. The homogenate was diluted with 10.0 ml of distilled water, shaken, and centrifuged. Two ml of the supernatant was added to 10.0 ml of distilled water. The potassium concentration of this final solution was assayed on the Coleman flame photometer after a standard curve had been obtained with known concentrations of potassium (0.04, 0.06, 0.08, 0.10, and 0.12 mEq/litre). The total potassium from each strip was calculated and expressed as mEq/kg wet weight.

The bathing fluid.

Except where it is specified in the results Krebs-Henseleit solution of the following composition was used: NaCl 118, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.1, $MgSO_4$ 1.2, $NaHCO_3$ 25, and glucose 11.0 mM.

Drugs.

Stock solutions of all drugs were made and kept at 4°C. The solutions of tyramine hydrochloride, histamine diphosphate, pronethalol hydrochloride (Alderlin, Ayerst), and ouabain were made in distilled water. Noradrenaline bitartrate was prepared in 0.01 N HCl. Phenoxybenzamine hydrochloride (Dibenzyline, S.K.F.) was dissolved in propylene glycol which had been acidified with 5 N HCl. For each experiment fresh dilutions with 0.9% NaCl were made from the stock solutions. In the case of noradrenaline and phenoxybenzamine one drop of 0.1 N HCl was added to

the dilution. The concentrations given in the text are the final concentrations in the bath; for noradrenaline, tyramine, and histamine these are in terms of the free base.

Reserpine was prepared by adding 100 mg to a mixture of 2.0 ml glacial acetic acid, 2.5 ml propylene glycol, and 2.5 ml ethanol. This was diluted with distilled water to 20.0 ml so that the final concentration was 5 mg/ml. Cats treated with reserpine were given 1.0 mg/kg twenty-four hours before the experiment.

Tests for statistical significance.

In most experiments tests were done between paired strips from the same cat, and statistical significance was determined by the t-test for paired observations (Goldstein, 1964). By this method t is equal to the mean difference divided by its standard error. When tests were done between strips from different cats statistical significance was determined by Student's t-test. All means are given with their standard errors. P values were obtained from a two tailed t-table (Steel and Torrie, 1960).

General procedure for experiments on dogs.

Mongrel dogs of either sex were injected with reserpine (0.5 mg/kg) intraperitoneally forty-eight and twenty-four hours before the experiment. They were anesthetized with pentobarbital sodium (20.0 mg/kg), and kept on positive pressure respiration with a Palmer Ideal Pump delivering room air (20 ml/kg) at sixteen strokes per minute through a tracheal cannula. The vagus nerves were cut at the level of the larynx in all dogs. Femoral arterial pressure was measured through a polyethylene catheter filled with 4% heparin solution in 0.9% NaCl and connected

to a Statham P-23 AC pressure transducer. The arterial pressure and the Lead II ECG were recorded on a Grass Polygraph. The heart rate was counted from the ECG. Body temperature was kept at 37°C by a heating lamp.

Vagal stimulation.

In one group of dogs, a cardiac rhythm originating from a subatrial pacemaker was elicited by stimulating the vagus nerve as follows: The peripheral stump of the cut right vagus was placed across a pair of platinum electrodes and carefully positioned in the neck to avoid stretching the nerve. Liquid petrolatum (U.S.P.) was poured into the neck incision so that it formed a pool around the nerve and the end of the electrodes. Square wave pulses one millisecond in duration were supplied by a Grass SD-5 stimulator. Threshold voltage was determined by briefly stimulating the nerve at a frequency of 20 pulses/second and gradually increasing the voltage (starting at 1 volt) until cardiac slowing was seen on the ECG. To elicit a subatrial rhythm the vagus was stimulated for ninety seconds at twice the threshold voltage and at a frequency of 20 pulses/second. The stimulation caused a marked bradycardia which was followed by a period of cardiac arrest; then vagal escape occurred and the P wave was always absent from the ensuing rhythm. The absence of the P wave was taken as evidence of a subatrial pacemaker. The subatrial pacemaker rate was determined by counting every beat during the last thirty seconds.

Drugs.

All drugs except reserpine were diluted in 0.9% NaCl and injected intravenously. Pronethalol was given either by rapid injection or

by infusion. Ouabain was injected over a one minute period.

Tests for statistical significance.

Tests for statistical significance within groups (experiments in which each dog served as its own control) were done by the t-test for paired observations. Comparisons between groups were made by Student's t-test. The means reported are given with their standard errors.

RESULTS

A. GENERAL CHARACTERISTICS OF THE OUABAIN-INDUCED CONTRACTION IN THE SPLEEN STRIP.

Experiments were done on sixteen strips, with each strip taken from a different spleen, to determine whether ouabain would cause contraction. All strips were kept in Krebs-Henseleit solution for four hours. Six were exposed for this time to 0.05 μg/ml of ouabain and another six to 0.1 μg/ml; the remaining four served as untreated controls. Neither the control strips nor the strips in the lower concentration of ouabain contracted, but three of the strips exposed to 0.1 μg/ml of ouabain contracted (3 to 6 mm). These observations indicated that the threshold concentration for ouabain-induced contraction was close to 0.1 μg/ml.

In subsequent experiments (over a hundred) we used concentrations of 0.3 μg/ml or greater. These doses always caused a marked contraction (56 to 144 mm). The contraction was sustained, requiring two to four hours for complete relaxation. There was a long latent period between the addition of ouabain to the bath and the onset of contraction. These features are illustrated in Figure 1.

Two groups of experiments were carried out to determine whether the contraction caused by ouabain was reproducible. In the first group (four experiments on sixteen strips) each strip was exposed to the same concentration of ouabain twice. The second exposure was made ten to twenty minutes after the contraction caused by the first exposure had completely disappeared. The concentration of ouabain varied in different experiments from 0.3 to 9.0 μg/ml. In all sixteen strips the second response was considerably less than the first. The mean decrease was 63.6 ± 9.6%, and there was no correlation between the concentration of ouabain and the amount by which the second response was reduced.

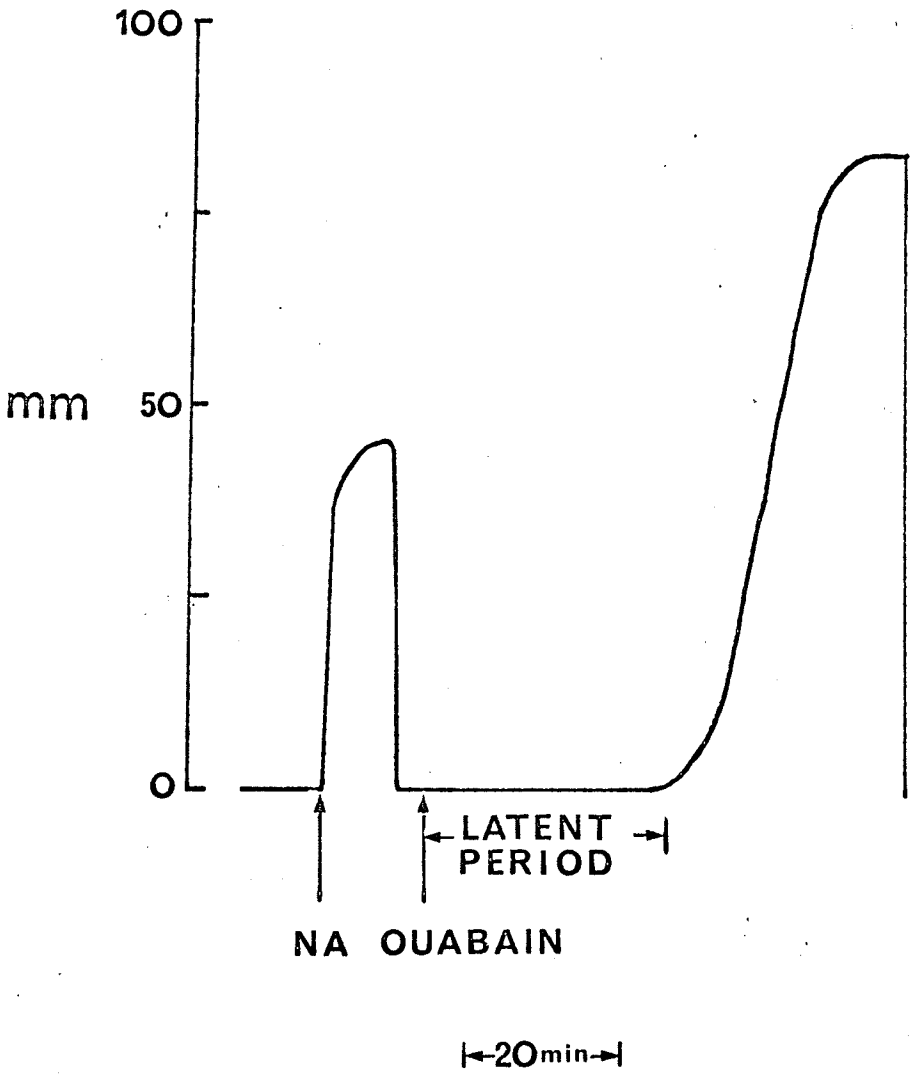

Figure 1. Contractile response of the isolated spleen strip to ouabain. NA, noradrenaline (1.0 µg/ml), ouabain (1.0 µg/ml).

In the second group of four experiments, each of four strips taken from one spleen was exposed once to the same concentration of ouabain (either 3.0 or 6.0 μg/ml). Although there was variation among strips taken from different spleens, the responses of strips taken from the same spleen were very similar (Table 1).

1. An attempt to correlate the amplitude of contraction with the concentration of ouabain.

The lack of reproducibility of responses to ouabain within a single strip ruled out the usual dose-response type experiment of exposing one tissue to graded concentrations of the agonist. For this reason we tried to correlate the amplitude of contraction to concentration by exposing each of four strips taken from the same spleen to a different concentration of ouabain. To test the feasibility of this technique four experiments were done in which each strip from an individual spleen was tested with a different concentration of noradrenaline. In all four experiments the amplitude of contraction was positively correlated with the concentration of noradrenaline; a typical experiment is shown in Figure 2.

However, when similar experiments were done with graded concentrations of ouabain on thirty-two strips taken from eight spleens, there was no correlation of contractile amplitude with concentration. These experiments are illustrated in Figure 3.

2. An attempt to correlate the amplitude of contraction with exposure time.

Six experiments were done to determine whether the length of time that the tissue was exposed to ouabain could be correlated with the amplitude of contraction. The same concentration of ouabain (3.0 μg/ml)

TABLE 1

Comparison of contraction amplitudes among strips cut from the same spleen and exposed to the same concentration of ouabain.

Amplitude of contraction in millimeters

Spleen No.	Ouabain µg/ml	Strip 1	Strip 2	Strip 3	Strip 4	Mean ± S.E.
1	3.0	71	77	62	75	71.3 ± 3.3
2	3.0	50	52	82	51	58.7 ± 7.8
3	6.0	81	73	76	93	80.8 ± 4.4
4	6.0	105	107	112	109	108.3 ± 1.5

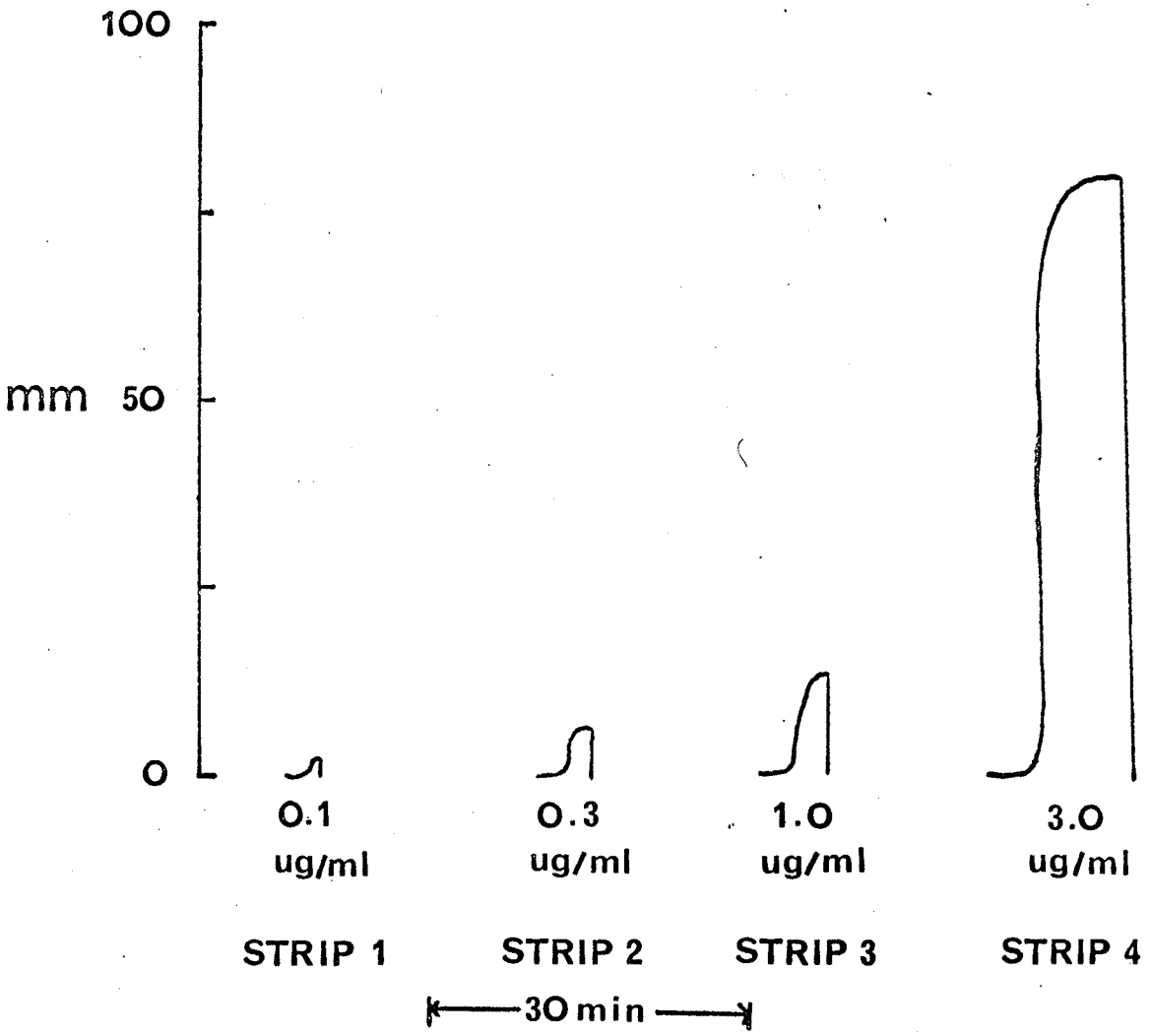

Figure 2. Responses of four strips from the same spleen to graded concentrations of noradrenaline.

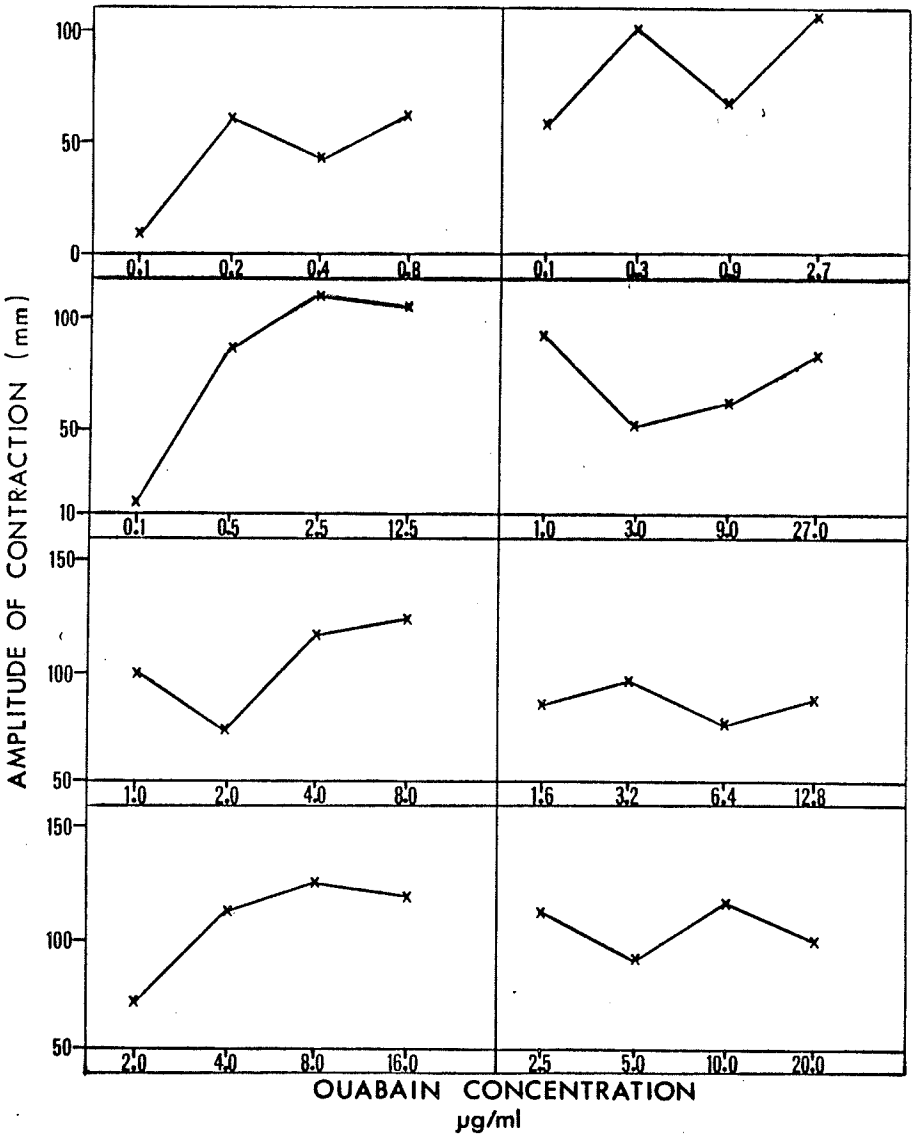

Figure 3. Responses to graded concentrations of ouabain. Each box represents the responses of four strips from the same spleen. Each point represents the response of one strip.

was used in all the experiments. Each experiment was on four strips from the same spleen, and each strip in an individual experiment was exposed to ouabain for a different time. In the first two experiments the minimum exposure time necessary to cause contraction was determined. The strips were exposed to ouabain for 5, 10, 15, and 20 minutes. The strips exposed for 20 minutes contracted (4 to 14 mm) 3 to 8 minutes after ouabain was washed out of the bath, but none of the strips exposed for less than 20 minutes contracted.

In the remaining four experiments two schedules of exposure times were used: 20, 23, 26 and 29 minutes in two experiments, and 20, 25, 30 and 35 minutes in the other two. Strips exposed for more than 23 minutes were contracting at the time ouabain was washed out of the bath, but contraction continued to reach a peak 10 to 30 minutes after the wash.

In two experiments there was a positive correlation between the amplitude of contraction and the length of exposure time (Figure 4, B and C). However, in the other two experiments this correlation held for only three strips (Figure 4, A and D).

3. Correlation of the latent period with the concentration of ouabain.

The latent period was taken as the time between the addition of ouabain to the bath and the beginning of contraction (Figure 1). This period was measured along with the contraction amplitude in the experiments described on page 24. The length of the latent period in strips cut from the same spleen and treated with the same concentration of ouabain was very consistent (Table 2). However, there was considerable variability among strips taken from different spleens (Table 2). In all eight experiments in which each strip from an individual spleen was

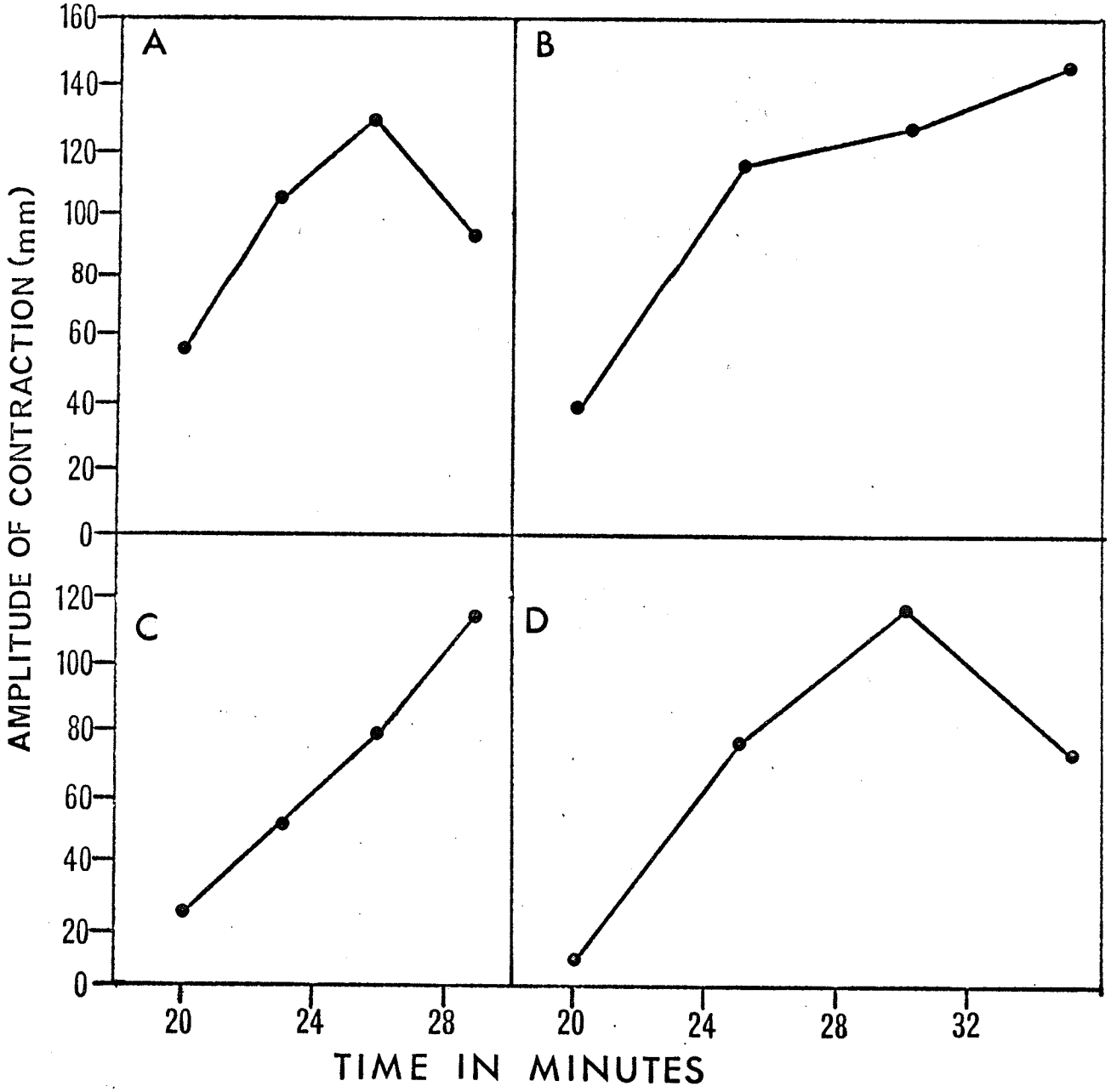

Figure 4. Variation in contractile amplitude with exposure time to ouabain (3.0 µg/ml). Each box represents the responses of four strips from the same spleen. Each point represents the response of one strip.

TABLE 2

Comparison of the latent periods among strips cut from the same spleen and exposed to the same concentration of ouabain.

Latent period in minutes

Spleen No.	Ouabain µg/ml	Strip 1	Strip 2	Strip 3	Strip 4	Mean ± S.E.
1	3.0	53	49	53	48	50.8 ± 1.3
2	3.0	35	27	33	34	32.3 ± 1.8
3	6.0	21	21	27	21	22.5 ± 1.5
4	6.0	25	25	30	30	27.5 ± 1.4

treated with a different concentration of ouabain (page 24) the length of the latent period was inversely related to the concentration, i.e. the higher the concentration the shorter the latent period (Figure 5). Again, the variability was large among strips exposed to the same concentration but cut from different spleens (Figure 5).

4. The effect of noradrenaline on the latent period.

Four experiments were done to determine whether noradrenaline would alter the latent period. Each experiment consisted of two strips from the same spleen. Both strips were first tested with noradrenaline and then exposed to ouabain (see Table 3-A for concentrations and exposure times). Ten to thirty minutes after giving ouabain, one strip was exposed for a second time to noradrenaline while ouabain was still in the bath, and both drugs were left in the bath until the contraction caused by ouabain reached maximum. The other strip served as a control. When noradrenaline was given in the presence of ouabain it initiated a contraction which consisted of two components. The first component was greater in amplitude (3 to 15 mm) but similar in shape to the response caused by the dose of noradrenaline given before ouabain. The second component of the response was similar in amplitude, shape, and duration to a ouabain-induced contraction (Figure 6). In all four experiments the second component occurred before the start of the ouabain-induced contraction in the control strip (Table 3-A). These experiments indicated that noradrenaline hastened the onset of the ouabain-induced contraction.

Two more experiments were done in the same way except histamine was used instead of noradrenaline (see Table 3-B for concentrations and exposure times). In contrast to noradrenaline, histamine had no effect on the latent period (Figure 7, Table 3-B).

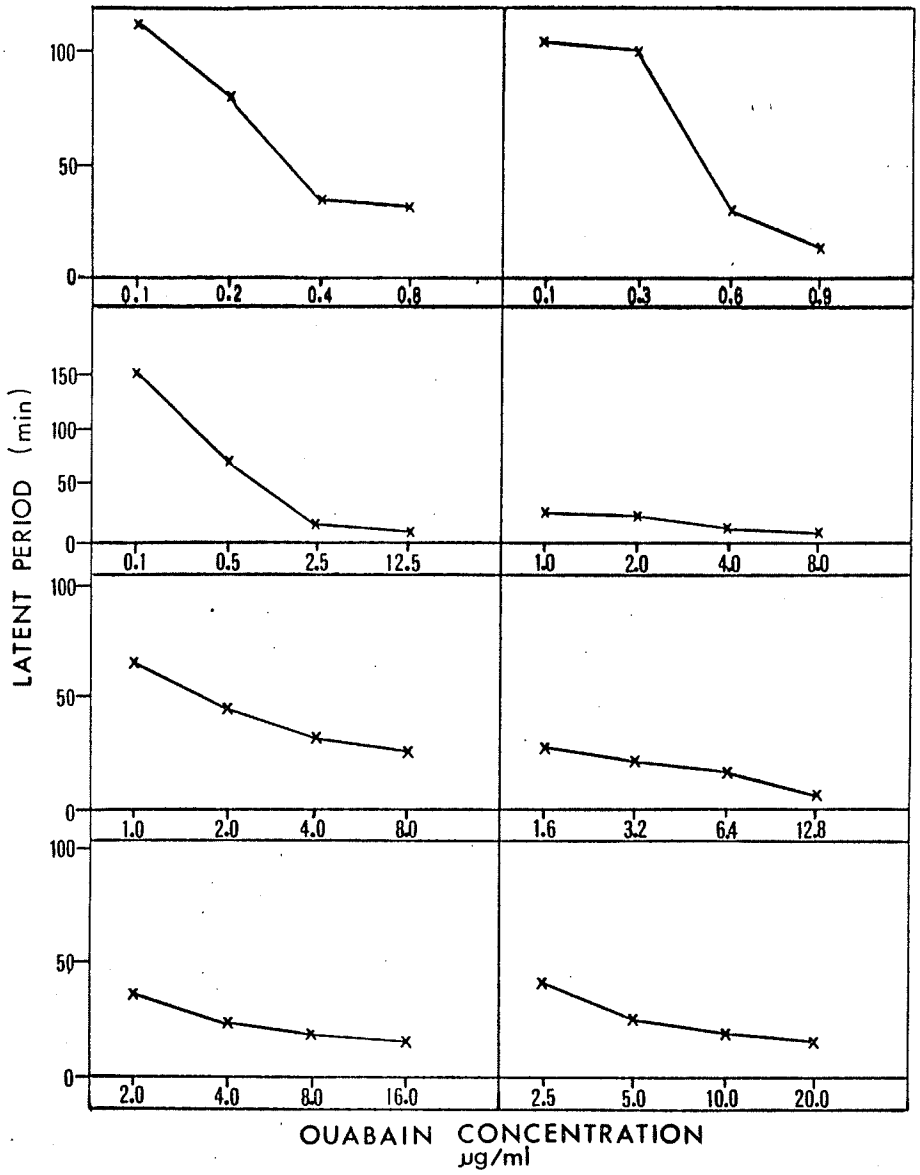

Figure 5. The inverse correlation between the latent period and the concentration of ouabain. Each box represents the responses of four strips from the same spleen. Each point represents the response of one strip.

TABLE 3

The effect of noradrenaline and histamine on the latent period.

A. The effect of noradrenaline

Spleen No.	Ouabain µg/ml	Noradrenaline µg/ml	1 Exposure minutes	Latent Period minutes Strip 1 with Noradrenaline	Strip 2 without Noradrenaline
1	0.5	0.5	31	36	40
2	0.5	0.5	32	37	45
3	3.0	5.0	7.0	12	24
4	3.0	5.0	7.0	15	34

B. The effect of histamine

Spleen No.	Ouabain µg/ml	Histamine µg/ml	1 Exposure minutes	Latent Period minutes Strip 1 with Histamine	Strip 2 without Histamine
1	0.5	5.0	29	78	78
2	0.5	5.0	21	104	101

1 The time between addition of ouabain to the bath and addition of either noradrenaline or histamine. Both drugs were left in the bath until the contraction caused by ouabain reached maximum.

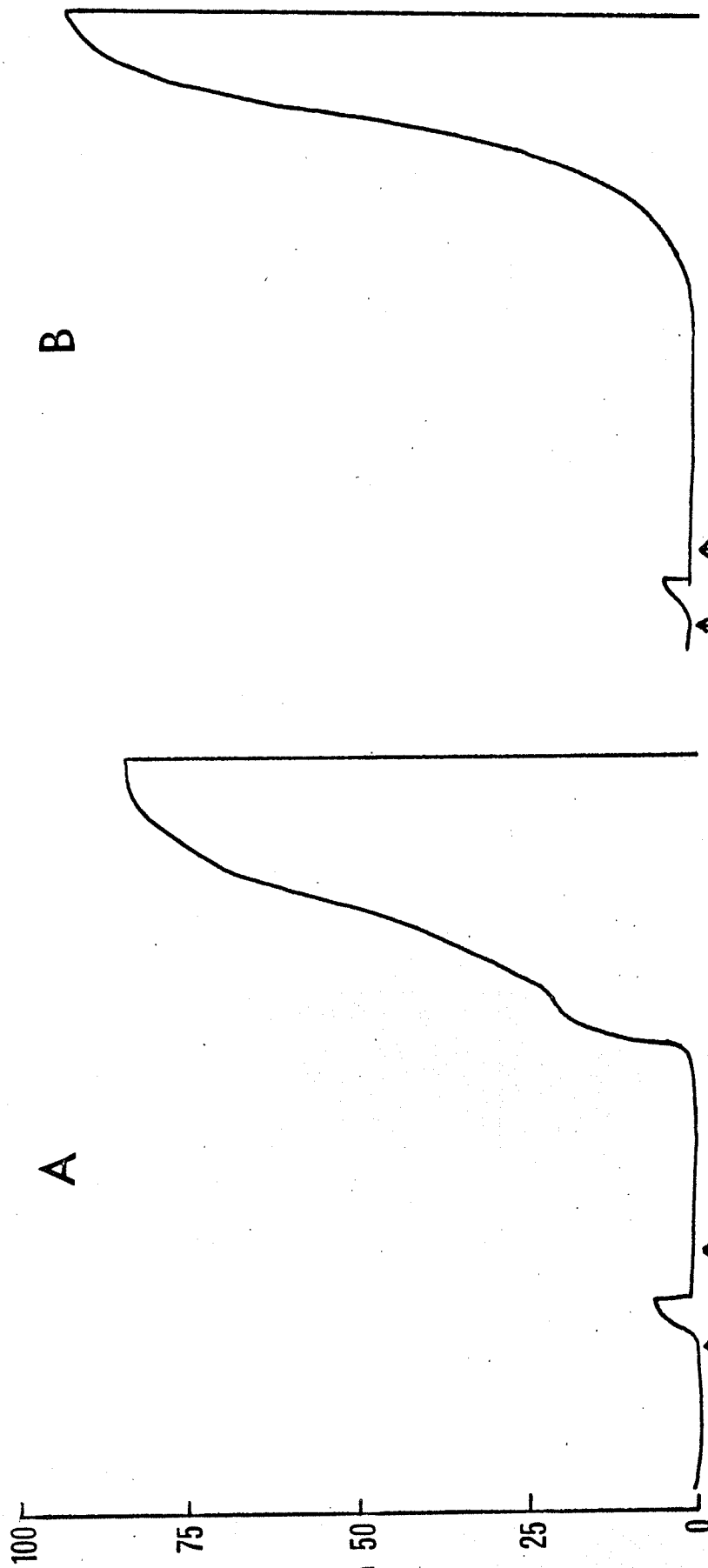

Figure 6. The effect of noradrenaline on the ouabain-induced contraction. Two strips, A and B, from the same spleen. NA, noradrenaline (0.5 µg/ml); OUA, ouabain (0.5 µg/ml). In A, the noradrenaline given after ouabain remained in the bath with ouabain until the second component of the contraction reached maximum.

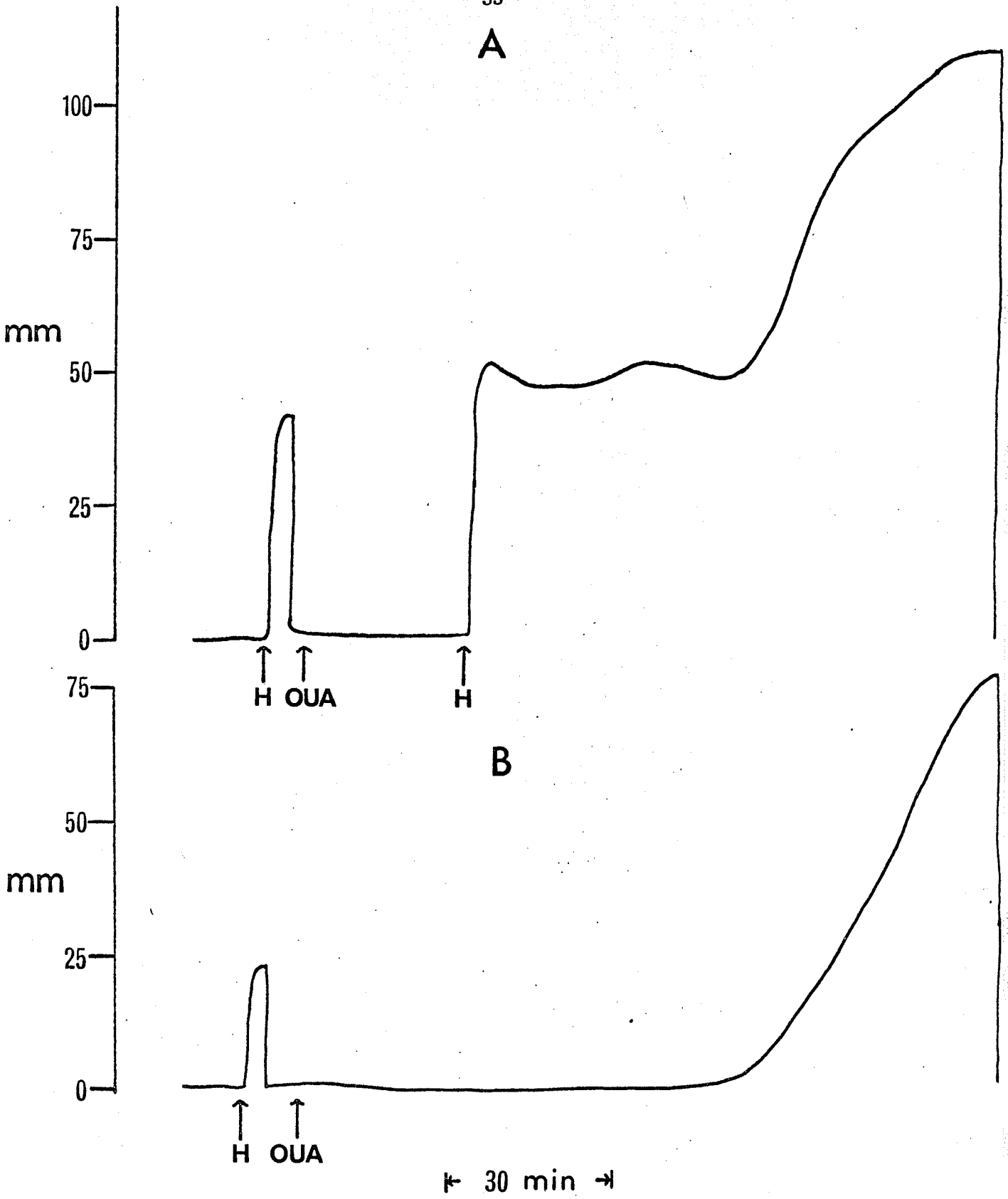

Figure 7. The effect of histamine on the ouabain-induced contraction. Two strips, A and B, from the same spleen. H, histamine (5.0 µg/ml); OUA, ouabain (0.5 µg/ml). In A, the histamine given after ouabain remained in the bath with ouabain until the second component of the contraction reached maximum.

B. EVIDENCE THAT THE OUABAIN-INDUCED CONTRACTION WAS DUE TO THE RELEASE OF NORADRENALINE

It was reasoned that if the ouabain-induced contraction was due to the release of noradrenaline, it should be inhibited by an α-adrenergic blocking agent, and it should be absent from strips depleted of noradrenaline either by pretreatment of the cats with reserpine or by chronic sympathetic denervation of the spleen.

1. The effect of the α adrenergic blocking agent phenoxybenzamine.

Five experiments were done, each with two strips cut from the same spleen. One strip was exposed for five minutes to phenoxybenzamine (1.0 μg/ml), and the other strip served as control. Five minutes after phenoxybenzamine was washed out of the bath, both strips were treated with ouabain (5.0 μg/ml). Phenoxybenzamine markedly reduced the response to ouabain in all five experiments. The mean amplitude of contraction for the control strips was 88.8 ± 7.7 mm, whereas the mean amplitude of contraction for strips treated with phenoxybenzamine was 12.8 ± 4.7 mm. The difference was statistically significant ($P < 0.01$).

2. The effect of reserpine pretreatment.

Experiments were done on sixteen strips taken from eight reserpine-pretreated cats, and on sixteen strips taken from eight untreated cats. Each strip was first exposed to noradrenaline (0.1 μg/ml in strips from reserpine-pretreated cats, and 0.5 μg/ml in normal strips). After the contraction elicited by noradrenaline had returned to baseline, each strip was exposed to tyramine (5.0 μg/ml), a drug known to release noradrenaline from the spleen (Innes, 1962). After exposure to tyramine each strip was given ouabain (5.0 μg/ml).

The results of these experiments are summarized in Figure 8. The strips from the reserpine-treated cats were more sensitive to noradrenaline than the strips from normal cats. This was expected, for it is well known that pretreatment with reserpine causes supersensitivity to catecholamines (Burn and Rand, 1958). The mean response of the reserpinized strips to noradrenaline was 34.3 ± 7.6 mm, whereas the mean response to noradrenaline in control strips was only 11.8 ± 3.1 mm even though they were exposed to a higher concentration of noradrenaline. The difference between the two groups was statistically significant by Student's "t" test ($P < 0.05$).

In contrast to noradrenaline, the responses to tyramine and ouabain were nearly abolished by reserpine pretreatment. In control strips the mean responses to tyramine and ouabain were 32.6 ± 6.7 mm and 126 ± 7.4 mm respectively. The mean responses to tyramine and ouabain in the strips from reserpine-treated cats were 1.2 ± 0.21 mm and 6.5 ± 2.6 mm respectively. The difference between the control and reserpine-treated groups was statistically significant ($P < 0.01$) for responses to both ouabain and tyramine.

The results of these experiments and those with phenoxybenzamine provided strong evidence that the contraction caused by ouabain in concentrations of 5.0 μg/ml and lower were due to the release of noradrenaline. To determine whether higher concentrations of ouabain would cause contraction in strips depleted of noradrenaline three more experiments were done on strips taken from cats pretreated with reserpine. Each experiment consisted of four strips taken from the same spleen, and each strip was exposed to a different concentration of ouabain. The concen-

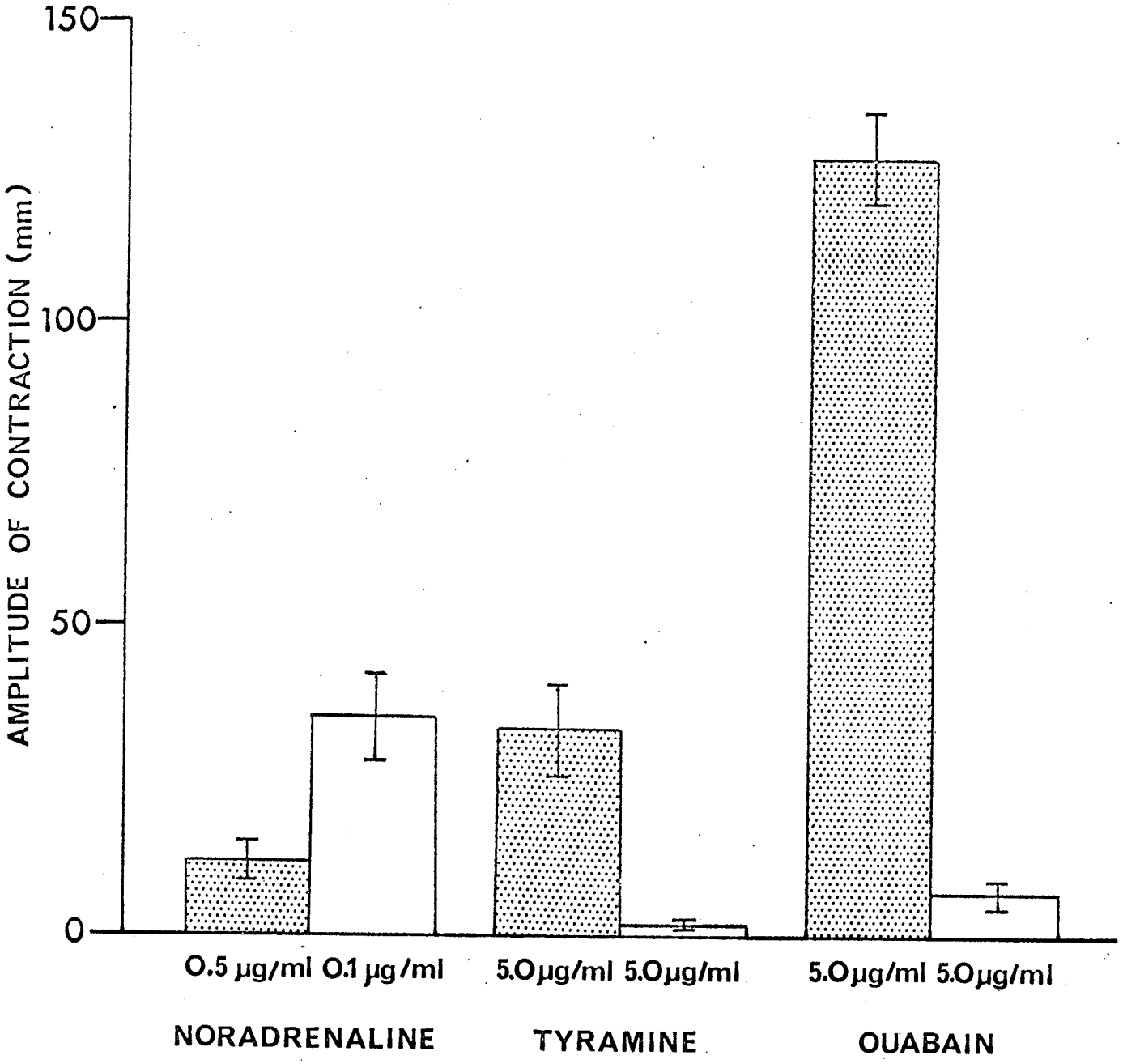

Figure 8. The effect of reserpine pretreatment on the ouabain-induced contraction. Open columns represent the mean response of sixteen strips from eight cats given reserpine (1.0 mg/kg) intraperitoneally 24 hours before the experiment. Stippled columns represent the mean response of sixteen strips from eight untreated cats. Bars at top of columns show the standard errors.

trations ranged from 1.0 μg/ml to 1.25 mg/ml (see Table 4).

The lowest concentration that caused a response comparable in magnitude (97 mm) to ouabain-induced contractions in strips not depleted of noradrenaline was 50.0 μg/ml, although in some cases lower concentrations caused small contractions (2 to 23 mm; Table 4). The responses of these strips were similar to strips not depleted of noradrenaline in that there was a long latent period which was inversely correlated with the concentration of ouabain, and there was only one experiment where the concentration was correlated with the amplitude of contraction (Table 4).

3. The effect of chronic denervation of the spleen.

Two experiments were done to establish that the source of noradrenaline for the ouabain-induced contraction was the postganglionic sympathetic nerve fibres. One strip was cut from each of four spleens; two of the spleens were denervated fourteen days before the experiment, and two were normally innervated controls. All four strips were tested with noradrenaline (0.1 μg/ml), tyramine (5.0 μg/ml), and ouabain (5.0 μg/ml).

The strips taken from denervated spleens were more sensitive to noradrenaline than the control strips; 0.1 μg/ml caused contractions of 76 and 60 mm in the two denervated strips but had no effect on the control strips. Tyramine, however, produced contraction in the control strips (29 and 39 mm), but had no effect on either of the denervated strips. Ouabain, like tyramine, caused contraction only in the control strips (80 and 125 mm). One of these experiments is illustrated in Figure 9. These experiments clearly showed that the source of noradrena-

TABLE 4

The effect of high concentrations of ouabain on spleen strips taken from cats pretreated with reserpine.

Spleen No.	Strip Number											
	Ouabain µg/ml	1 Contraction mm	Latent Period min	Ouabain µg/ml	2 Contraction mm	Latent Period min	Ouabain µg/ml	3 Contraction mm	Latent Period min	Ouabain µg/ml	4 Contraction mm	Latent Period min
1	1.0	2	35	5.0	11	20	25	23	15	125	73	10
2	2.7	6	32	8.0	7	20	24	2	15	72	2	13
3	10	0	-	50	97	7	125	101	3	1250	60	2

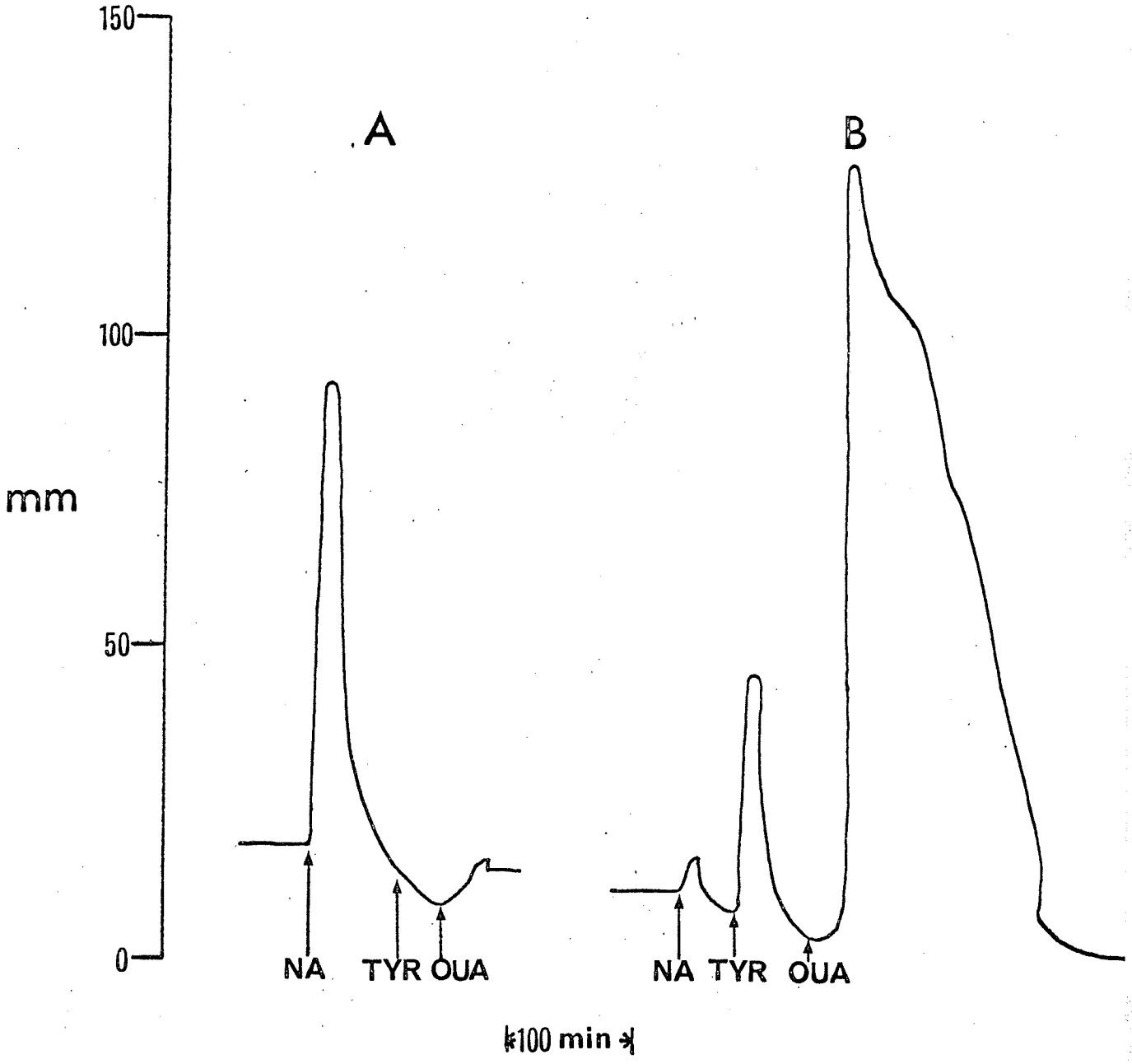

Figure 9. The effect of chronic denervation of the spleen on the ouabain-induced contraction. A, strip from spleen denervated fourteen days before the experiment. B, strip from normally innervated spleen. NA, noradrenaline (0.1 µg/ml), TYR, tyramine (5.0 µg/ml) OUA, ouabain (5.0 µg/ml).

line for the ouabain-induced contraction was the postganglionic adrenergic fibres.

C. PROTECTION OF NORADRENALINE RECEPTORS BY OUABAIN AGAINST PHENOXYBENZAMINE BLOCKADE.

It is known that noradrenaline receptors can be partially protected from phenoxybenzamine blockade if a large concentration of noradrenaline is present at the receptor sites when the tissue is exposed to phenoxybenzamine (page 72). However, it has never been shown that a drug which acts by release of noradrenaline, e.g. tyramine, can release a quantity sufficient to protect. Since it was suspected from the amplitude and duration of the ouabain-induced contraction that large amounts of noradrenaline were being released, six experiments were done to determine whether ouabain would afford any protection.

Each experiment consisted of four strips from the same spleen. All four strips were tested with noradrenaline (0.5 μg/ml) and histamine (0.5 μg/ml). After tests with noradrenaline and histamine, one strip was exposed to ouabain (7.0 μg/ml) and one was exposed to phenoxybenzamine (0.05 μg/ml) for five minutes. The third strip was exposed to both ouabain (7.0 μg/ml) and phenoxybenzamine (0.05 μg/ml); in this strip the phenoxybenzamine was added just as the ouabain-induced contraction reached maximum, then after five minutes both the ouabain and phenoxybenzamine were washed out of the bath. The fourth strip served as an untreated control.

The contractions caused by ouabain varied from 90 to 122 mm, and the time required for complete relaxation varied from 3 to 4 hours. At the end of this time all four strips were tested again with the same concentrations of noradrenaline and histamine. The contractions caused

by the second exposure to these drugs were expressed as a percentage of the contraction caused by the first exposure. To facilitate description of the results this percentage figure will be called the "remaining response". Figure 10 shows that the mean remaining responses for both noradrenaline and histamine were less than 100% in all four strips.

The mean differences in the remaining responses between the untreated control strips and the strips treated only with ouabain were statistically compared by paired data analysis. The mean differences between the strips treated with phenoxybenzamine only, and the strips treated with ouabain plus phenoxybenzamine were similarly compared. In the first comparison the responses to noradrenaline were 15.3 ± 8.1 less in the strips treated only with ouabain than in the control strips, but this was not statistically significant ($P > 0.1$). The responses to histamine in this comparison were 42 ± 17.9 less in the strips treated with ouabain which was of borderline statistical significance ($P < 0.06$).

In the comparison between strips treated with phenoxybenzamine only and the strips treated with phenoxybenzamine plus ouabain, the remaining responses to noradrenaline were 16.5±6.4 greater in the strips treated with ouabain plus phenoxybenzamine, and this was a statistically significant difference ($P < 0.05$). On the other hand, there was no significant difference in the responses to histamine. They were 0.4 ± 1.1 less in the strips treated with phenoxybenzamine plus ouabain than they were in the strips treated with phenoxybenzamine only ($P > 0.5$). Therefore, these data indicated that ouabain could partially protect noradrenaline receptors against phenoxybenzamine blockade (Figure 10), and that the protection was specific since the histamine receptors were not protected.

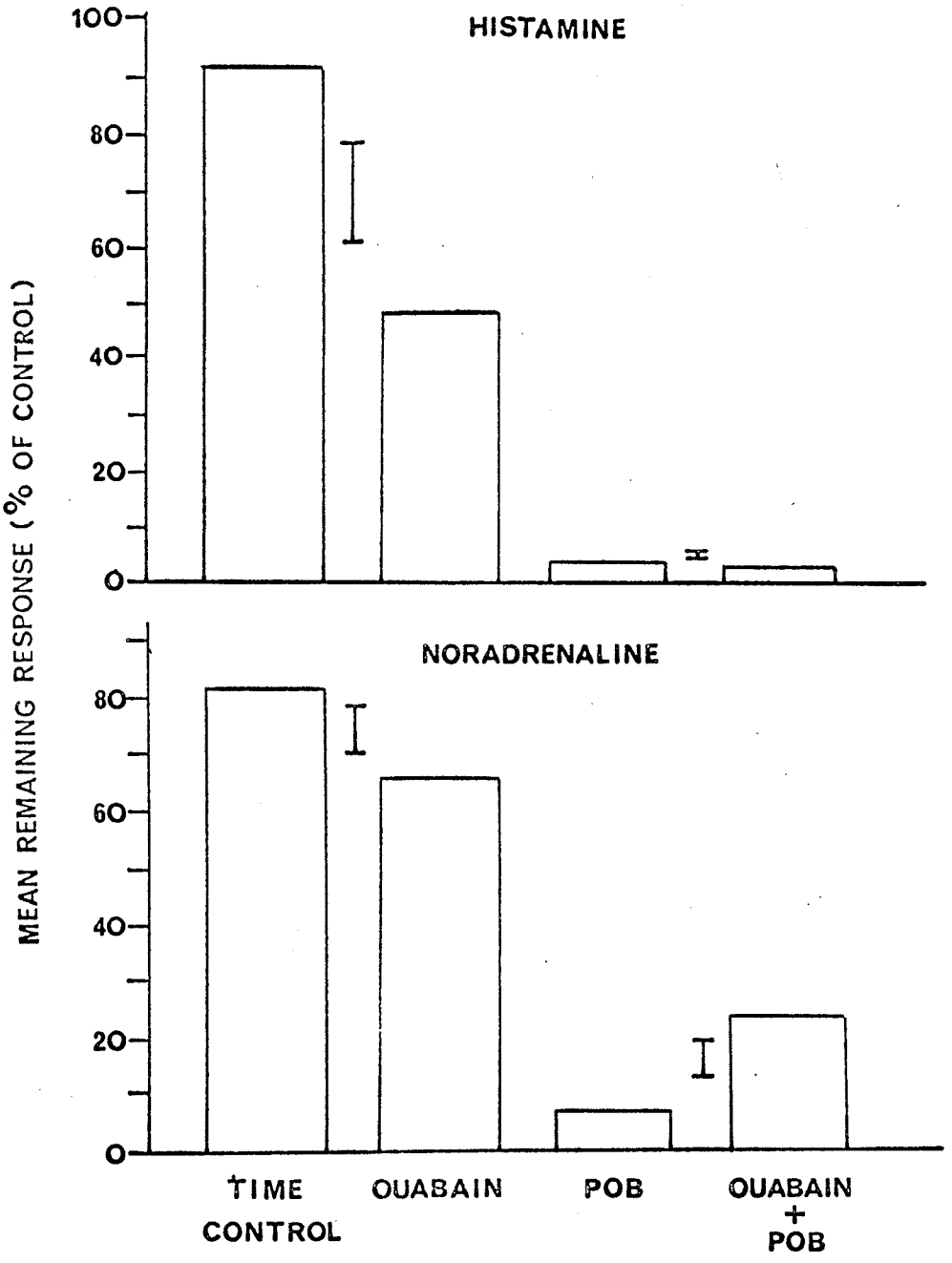

Figure 10. Protection of noradrenaline receptors by ouabain against phenoxybenzamine blockade. Each column represents the mean response of strips from six spleens to noradrenaline (0.5 μg/ml) or histamine (0.5 μg/ml) 3-4 hours after exposure to the drugs indicated. Bars between columns show standard errors of the mean difference. TIME CONTROL, no drugs given between tests with noradrenaline or histamine. OUABAIN (7.0 μg/ml), POB, phenoxybenzamine (0.05 μg/ml, 5 min exposure), OUABAIN + POB, ouabain (7.0 μg/ml) + phenoxybenzamine (0.05 μg/ml, 5 min exposure).

However, it was not possible to tell whether the protection afforded by ouabain was due to the noradrenaline release or to a direct effect of ouabain on the noradrenaline receptors. To test whether the latter was a tenable possibility six experiments were done on strips from spleens depleted of noradrenaline by pretreatment of the cats with reserpine. The experimental design was the same as before, and the results were analyzed in the same way. Figure 11 illustrates the results.

A notable finding in these experiments was that ouabain potentiated noradrenaline. The mean remaining responses to noradrenaline were 31.2 $\pm$ 8.8 greater in the strips treated with ouabain alone than in the untreated control strips, and this difference was statistically significant ($P < 0.02$). The remaining responses to histamine were 15.3 $\pm$ 26.6 greater in the ouabain treated strips than in the control strips, which was not statistically significant ($P > 0.5$). In the strips treated with ouabain plus phenoxybenzamine the responses to noradrenaline were 4.5 $\pm$ 1.3 greater than in the strips treated with phenoxybenzamine only, and the difference was statistically significant ($P < 0.05$). In the case of the responses to histamine, the strips treated with ouabain plus phenoxybenzamine were 3.9 $\pm$ 10.6 greater than in the strips treated with phenoxybenzamine only; this was not statistically significant ($P > 0.5$).

The potentiating effect of ouabain was more than enough to account for the difference in the remaining response to noradrenaline between the strips treated with ouabain plus phenoxybenzamine and the strips treated with phenoxybenzamine only. Also, this difference was only about one fourth as great in strips from reserpine-pretreated cats as it was in normal strips. Therefore, it was concluded that the protection of noradrenaline receptors found in the normal strips was due to the release of

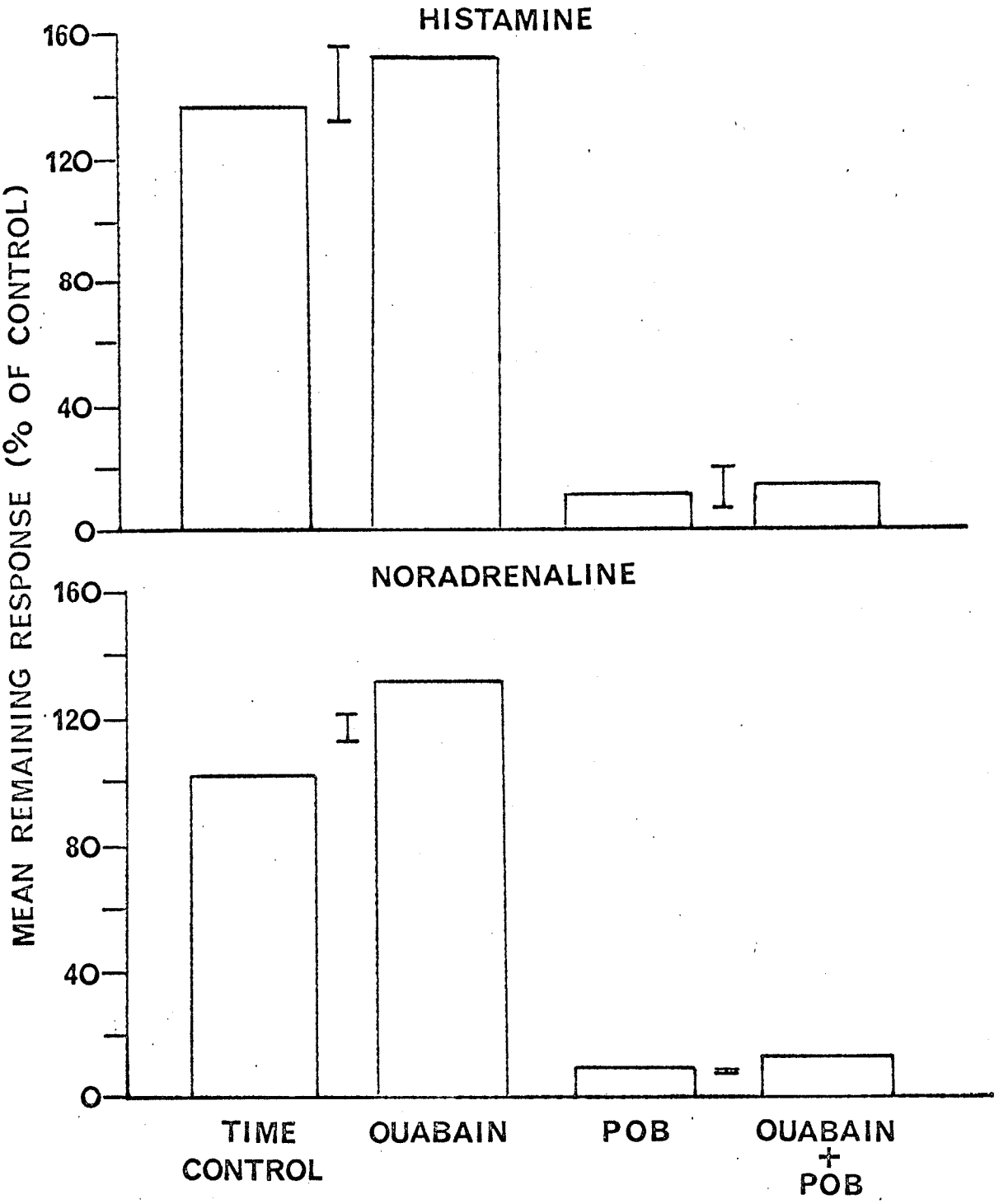

Figure 11. Failure of ouabain to protect noradrenaline receptors against phenoxybenzamine blockade in strips from cats given reserpine (1.0 mg/kg) 24 hours before the experiment. Each column represents the mean response to noradrenaline (0.5 μg/ml) or histamine (0.5 μg/ml) 3 hours after exposure to the drugs indicated. Bars between columns show standard errors of the mean difference. TIME CONTROLS, no drugs given between tests with noradrenaline or histamine, OUABAIN (7.0 μg/ml), POB, phenoxybenzamine (0.05 μg/ml, 5 min exposure), OUABAIN + POB, ouabain (7.0 μg/ml) + phenoxybenzamine (0.05 μg/ml, 5 min exposure).

noradrenaline.

To determine whether the potentiation observed in these experiments would occur over a range of noradrenaline concentrations, four more experiments were done on spleens from cats pretreated with reserpine. One strip from each spleen was exposed to cumulative concentrations of noradrenaline. That is, when the contraction caused by the first exposure to noradrenaline (0.01 μg/ml) had reached maximum, 0.05 μg/ml more was added, bringing the cumulative concentration to 0.06 μg/ml; after the strip had responded maximally the larger doses were added. However, since noradrenaline rapidly deteriorates at pH 7.4 the effective concentration at any given time could not be known exactly. For this reason the effective concentrations are arbitrarily given as the increments added rather than the cumulative concentrations.

Sixty minutes after the first exposure to noradrenaline, the strips were treated with ouabain (5.0 μg/ml) for fifteen minutes. They were then washed and exposed a second time to the same concentrations of noradrenaline. In all four experiments ouabain potentiated the response to noradrenaline at each concentration. This can be seen by the shift in the dose-response curve to the left in Figure 12. We did not test the effect of time alone by obtaining two consecutive dose-response curves without ouabain, because in the protection experiments time alone had no effect on the responses to noradrenaline in strips from reserpine-pretreated cats (Figure 11).

D. THE EFFECT OF PRONETHALOL ON THE OUABAIN-INDUCED CONTRACTION.

Since we had previously found that pronethalol antagonized some of the cardiac actions of ouabain (Tuttle and Innes, 1966), it was

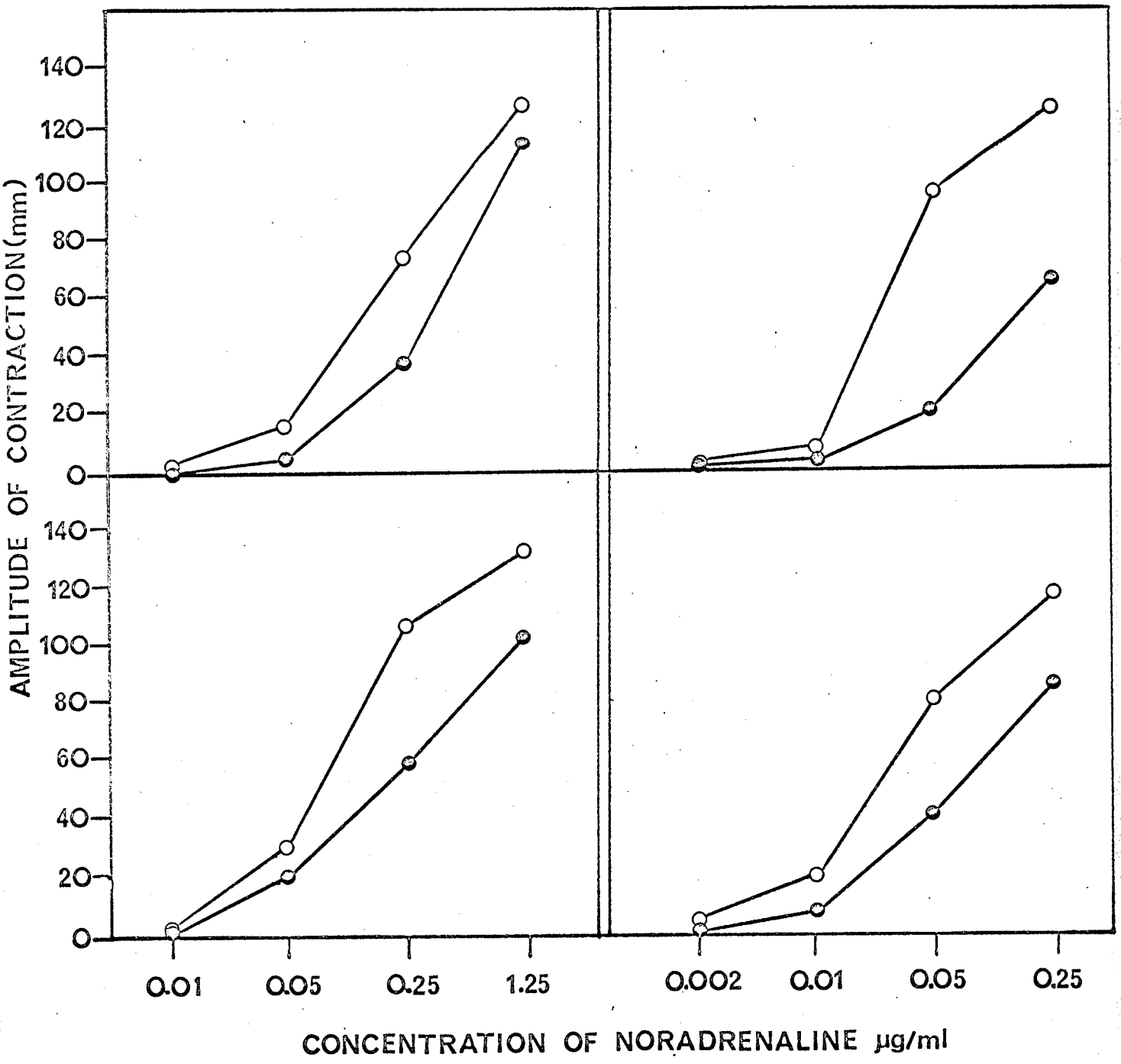

Figure 12. Potentiation of noradrenaline by ouabain in strips from cats given reserpine (1.0 mg/kg) 24 hours before the experiment. Filled circles, responses to noradrenaline before ouabain. Open circles responses to noradrenaline after ouabain (5.0 µg/ml).

of interest to determine whether pronethalol had any effect on ouabain's ability to release noradrenaline. Eight experiments were done, each on two strips cut from the same spleen. One strip was treated with pronethalol (10.0 μg/ml) for fifteen minutes, and the other strip served as an untreated control. Both strips were then exposed to ouabain (10.0 μg/ml). In all eight experiments pronethalol reduced the amplitude of the ouabain-induced contraction (Figure 13). The mean difference in the responses between the pronethalol-treated and control strips was 53.2 ± 10.9 mm, and this was statistically significant ($P < 0.01$).

To determine whether the attenuated response to ouabain was due to a decreased release of noradrenaline or to a reduced sensitivity of the smooth muscle to noradrenaline, four more experiments were done. As before, each experiment consisted of two strips from the same spleen. Both strips were tested with noradrenaline (0.5 μg/ml). Then one strip was exposed to pronethalol (10.0 μg/ml) for fifteen minutes, while the other strip served as control. Both strips were then tested a second time with noradrenaline (0.5 μg/ml). The response to the second exposure of noradrenaline was expressed as a percentage of the first response. In the control strips the second response was always reduced (Figure 13). However, in the pronethalol-treated strips the second response was always greater than the first response (Figure 13). The mean difference between the control and the pronethalol-treated strips was 39.2 ± 8.2 and this was statistically significant ($P < 0.05$).

Since pronethalol enhanced rather than inhibited the response to exogenous noradrenaline, it was concluded that the attenuated response to ouabain in the presence of pronethalol was due to a decreased release

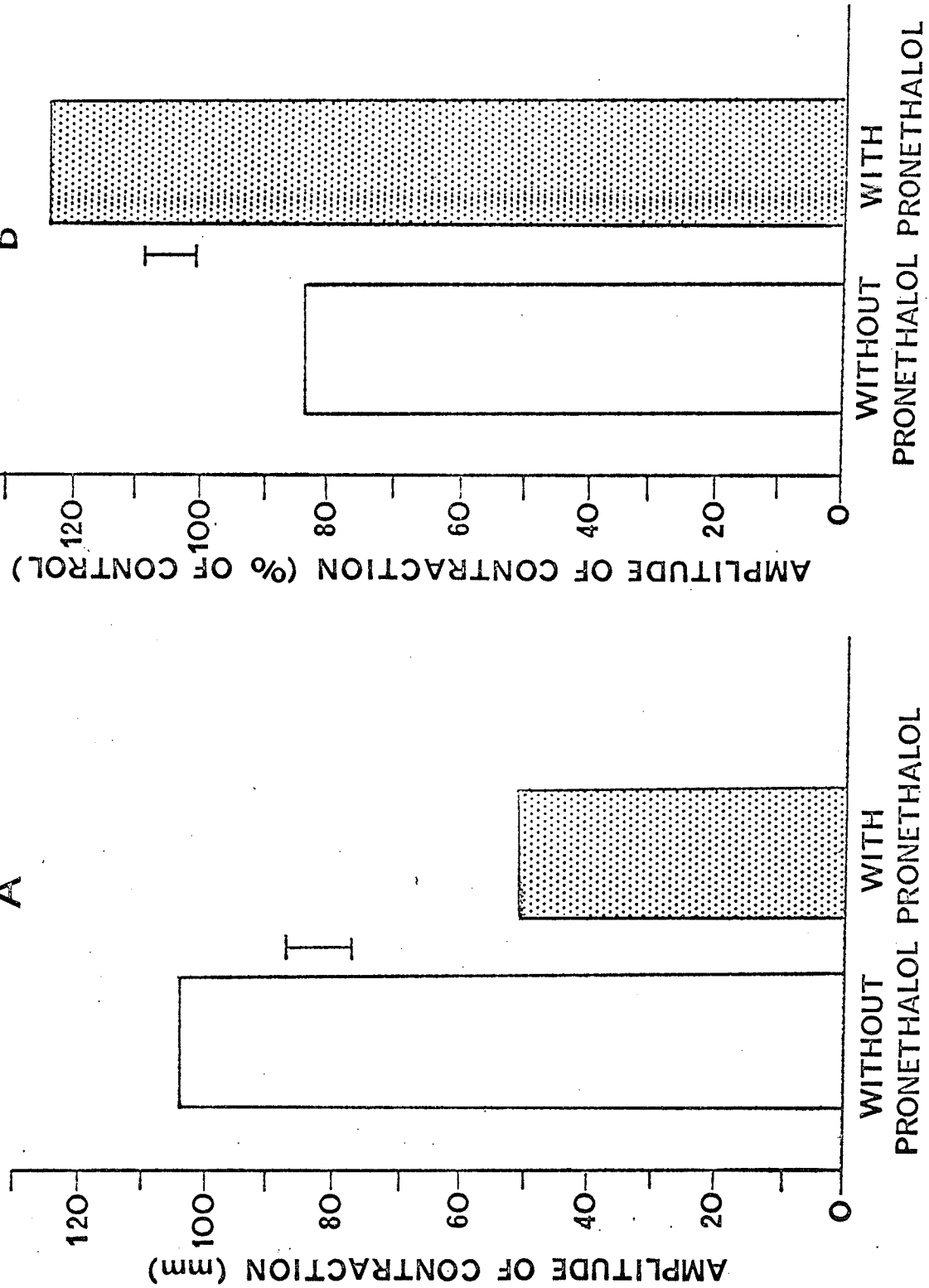

Figure 13. The effect of pronethalol on the ouabain-induced contraction. A, open column, mean response of strips from eight spleens to ouabain (10.0 µg/ml), stippled column, mean response of strips from same eight spleens to ouabain (10.0 µg/ml) after pronethalol (10.0 µg/ml). B, open column, mean response of strips from four strips to noradrenaline (0.5 µg/ml), stippled column, the mean response of strips from the same four spleens to noradrenaline (0.05 µg/ml) after pronethalol (10.0 µg/ml). Bars between columns show standard errors of the mean difference.

of noradrenaline.

E. THE EFFECT OF OUABAIN ON TISSUE POTASSIUM.

It is known that cardiac glycosides cause downhill ion movement (loss of intracellular potassium and gain of sodium) in many tissues (see page 14). For this reason eighteen experiments were done on seventy-two strips to determine whether downhill ion movement was associated with the release of noradrenaline caused by ouabain. The contraction amplitude and changes in total tissue potassium were used as indexes of catecholamine release and downhill ion movement. Since pronethalol opposed the release of noradrenaline by ouabain, it was included in these experiments to determine whether it would also oppose downhill ion movement caused by ouabain. Each experiment consisted of four strips cut from the same spleen. One strip was treated with ouabain (3.0 μg/ml), one with pronethalol (10.0 μg/ml), and one with pronethalol (10.0 μg/ml) plus ouabain (3.0 μg/ml). The fourth strip served as an untreated control. The experiments were divided into three groups with six experiments in each group. Each group was treated in the same way except that the first group was exposed to the drugs for fifteen minutes, while the second and third groups were exposed to the drugs for thirty and forty-five minutes respectively. The strips given both pronethalol and ouabain were an exception to these exposure times in that pronethalol was added to the bath five minutes before ouabain.

The results of these experiments are summarized in Table 5. There was no contraction in any of the strips exposed for fifteen minutes. However, ouabain by itself caused a loss of tissue potassium. The mean difference in potassium content between control strips and strips with

TABLE 5

Delay of the ouabain-induced loss of potassium and contraction by pronethalol.

Exposure	Control		1 Ouabain Alone		2 Pronethalol Alone		3 Ouabain + Pronethalol	
min	K^+ Content mEq/kg	Contraction mm	K^+ Content mEq/kg	Contraction mm	K^+ Content mEq/kg	Contraction mm	K^+ Content mEq/kg	Contraction mm
15	76.1 ± 5.1	0	65.9 ± 3.5	0	75.3 ± 4.5	0	73.6 ± 3.7	0
30	73.5 ± 2.9	0	63.0 ± 2.9	40.5 ± 9	82.8 ± 8.2	0	69.4 ± 1.4	1.0 ± 0.6
45	80.0 ± 4.5	0	70.5 ± 3.2	78.5 ± 9.7	77.8 ± 3.3	0	67.4 ± 3.6	34.8 ± 11.2

1 Ouabain (3.0 µg/ml)

2 Pronethalol (10.0 µg/ml)

3 Ouabain (3.0 µg/ml), pronethalol (10.0 µg/ml)

ouabain alone was 10.2 ± 3.6 mEq/kg. This was statistically significant ($P < 0.05$). There was no significant difference between the control strips, and either the strips treated with ouabain plus pronethalol or the strips treated with pronethalol alone (Table 5).

Ouabain given alone caused contraction in all of the strips exposed for thirty minutes. The mean amplitude of contraction was 40.5 ± 9.0 mm. This figure is small in comparison to the usual contraction caused by ouabain because in three experiments the tissues were still contracting at the end of the thirty minute period, when they were removed from the bath. Only two of the strips treated with ouabain plus pronethalol had even started to contract by the end of the thirty minute period. The average amplitude of contraction was 1.0 ± 0.6 mm. In this group ouabain given alone caused a loss of tissue potassium in every experiment. The mean difference between control strips and strips exposed to ouabain only was 10.5 ± 3.2 mEq/kg. This was statistically significant ($P < 0.05$). There was no statistically significant difference between control strips and either the ouabain plus pronethalol treated strips or the strips treated with pronethalol alone (Table 5).

In the group exposed for forty-five minutes ouabain given alone caused contraction in every strip, and each strip had reached its maximum before the end of the forty-five minute period. The average contraction amplitude was 78.5 ± 9.7 mm. In the strips treated with ouabain plus pronethalol four were still contracting when they were removed from the bath at the end of the forty-five minute period; the other two had already reached maximum. The mean amplitude of contraction was 34.8 ± 11.2 mm. There was a significant loss in tissue potassium both in the strips treated with ouabain alone and in the strips treated with proneth-

alol plus ouabain. The mean difference between control strips and ouabain-treated strips was 9.5 ± 1.9 mEq/kg. The mean difference between control and ouabain plus pronethalol treated strips was 12.6 ± 1.8 mEq/kg. In both cases the differences were statistically significant with $P < 0.01$. There was no significant difference between control and strips treated with pronethalol alone (Table 5).

F. THE EFFECT OF AN INCREASED CALCIUM CONCENTRATION ON THE AMPLITUDE OF CONTRACTION AND THE LATENT PERIOD.

The above experiments showed that ouabain caused downhill ion movement, and that this preceded the release of noradrenaline as judged by the onset of contraction. The possibility of a causal relationship was suggested inasmuch as pronethalol delayed both downhill ion movement and contraction. It was reasoned that if downhill ion movement was responsible for noradrenaline release, a procedure known to oppose ionic shifts should also oppose the release of noradrenaline. For example, it has been shown that calcium influences the passage of sodium through the cell membrane, and that increasing the extracellular calcium concentration decreases the permeability of the cell to sodium. This is the basis of the membrane stabilization effect of calcium (see page 77).

Therefore six experiments were done to determine the effect of an increased extracellular calcium concentration on the amplitude and latent period of the ouabain-induced contraction. Each experiment consisted of two strips from the same spleen. One was equilibrated for sixty minutes in Krebs-Henseleit solution containing the usual concentration of calcium (2.5 mM) and the other strip was equilibrated for the same period in Krebs-Henseleit solution containing twice the usual concentration of calcium (5.0 mM). Both strips were tested with noradrena-

line (1.0 μg/ml); then both were exposed to ouabain (10.0 μg/ml).

Figure 14 shows that even though the sensitivity of the tissue to noradrenaline was enhanced by increasing the calcium concentration, the response to ouabain was antagonized. The mean response to exogenous noradrenaline was 15.7 ± 3.1 mm greater in the strips bathed in the high calcium concentration than in the control strips ($P < 0.01$). In contrast, the mean response to ouabain was 9.0 ± 3.2 mm less in the strips in high calcium than in the control strips ($P < 0.05$). However, the most striking change caused by increasing the calcium concentration was that ouabain took significantly longer to act. The mean increase in the latent period was 8.0 ± 2.9 minutes ($P < 0.05$).

These results indicated that the noradrenaline-releasing action of ouabain was inhibited by increasing the calcium concentration.

G. THE EFFECT OF A LOW EXTRACELLULAR SODIUM CONCENTRATION ON THE LATENT PERIOD.

If the inhibition of ouabain in the above experiments was due to a decreased permeability to sodium and thus a decreased downhill ion movement, then reducing the amount of sodium available to run downhill should also inhibit ouabain. However, in these experiments the contraction amplitude could not be used as an index of altered noradrenaline release because it was later found that sensitivity of the spleen strip to noradrenaline was impaired by reducing the extracellular sodium concentration. Therefore, four experiments were done to determine whether a low sodium concentration would have an effect on the latent period similar to that caused by the high calcium concentration.

As before, each experiment was on two strips from the same spleen. The control strip was equilibrated for sixty minutes in Krebs-

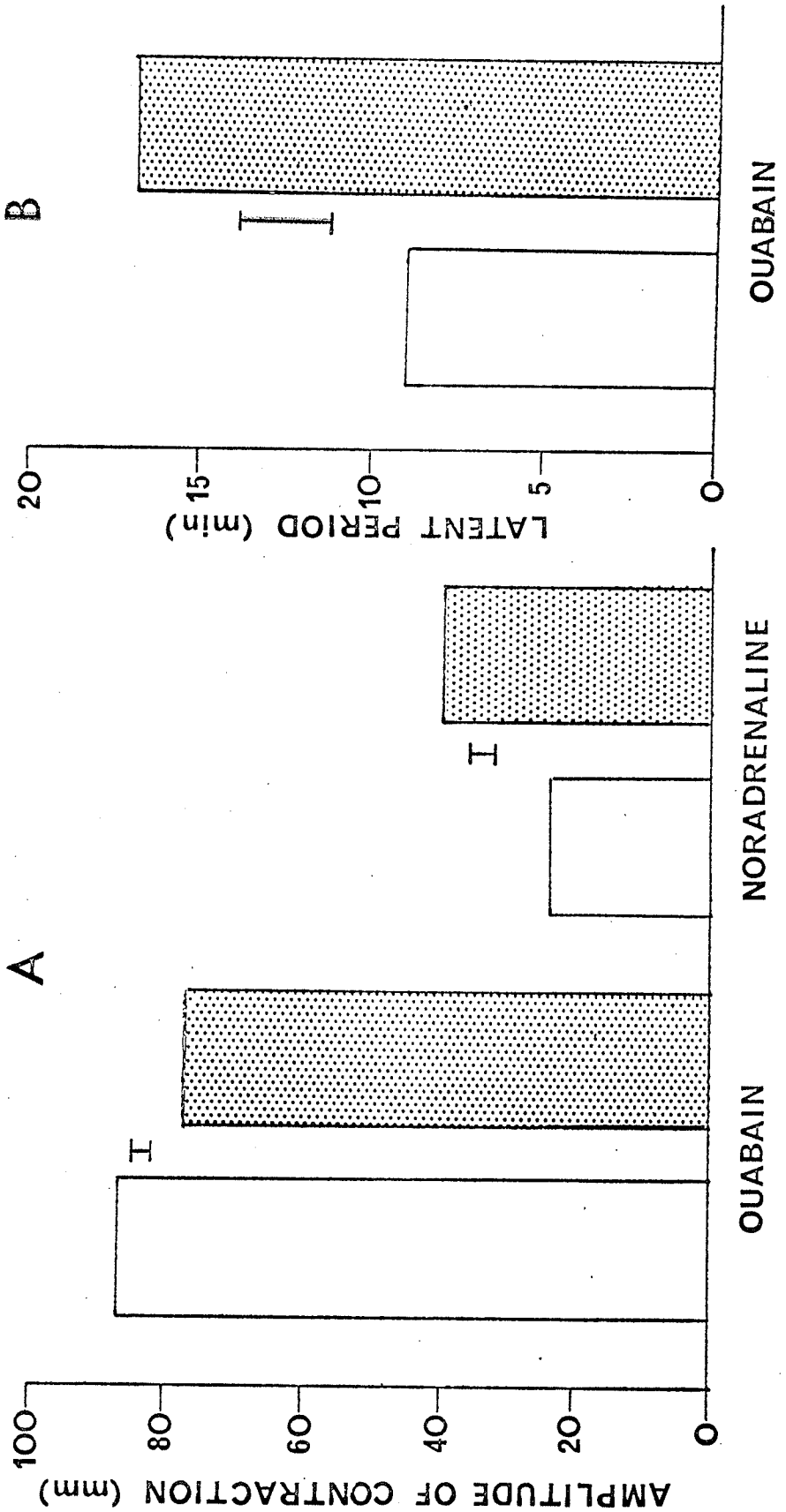

Figure 14. The effect of increased extracellular calcium concentration on the response to ouabain. A, amplitude of contractions due to ouabain (10.0 µg/ml), and noradrenaline (1.0 µg/ml). B, ouabain latent period. Open columns, mean response of strips from six spleens bathed in Krebs-Henseleit solution containing 2.5 mM calcium. Stippled columns, mean response of strips from the same six spleens, bathed in Krebs-Henseleit solution containing 5.0 mM calcium. Bars between columns show standard errors of the mean difference.

Henseleit solution containing the usual amount of NaCl (118 mM) and $NaHCO_3$ (25 mM). The experimental strip was equilibrated for the same time in a solution in which the NaCl was replaced by sucrose (236 mM). Otherwise the two solutions were the same. At the end of the sixty minute period both strips were tested with noradrenaline (1.0 μg/ml). Both were then exposed to ouabain (3.0 μg/ml).

It is apparent from Figure 15 that reducing the sodium concentration markedly increased the latent period. The mean difference between control and experimental strips was 17.0 ± 4.6 minutes, and this was statistically significant ($P < 0.05$). The contraction amplitude for both exogenous noradrenaline and ouabain was significantly less in the strips bathed in low sodium solution than in the strips bathed in the usual sodium concentration (Figure 15). The mean difference between the control and experimental strips for the responses to noradrenaline was 26.0 ± 5.4 mm ($P < 0.02$), and for the responses to ouabain the mean difference was 55.5 ± 9.1 mm ($P < 0.01$).

H. THE EFFECT OF A DEPOLARIZING CONCENTRATION OF EXTRACELLULAR POTASSIUM.

It is known that smooth muscle still responds to many agonists even when it is bathed in media containing depolarizing concentrations of potassium (Evans, Schild and Thesleff, 1958). Therefore six experiments were done to determine whether ouabain was similar to other agonists in this regard. Each experiment consisted of two strips from the same spleen. Two different bathing media were used. The first was a control solution similar to Krebs-Henseleit except that it contained no calcium, and ethylenediaminetetraacetic acid (EDTA) (0.1 mg/ml) was added. The experimental solution differed from the control solution in that KCl (118 mM)was substi-

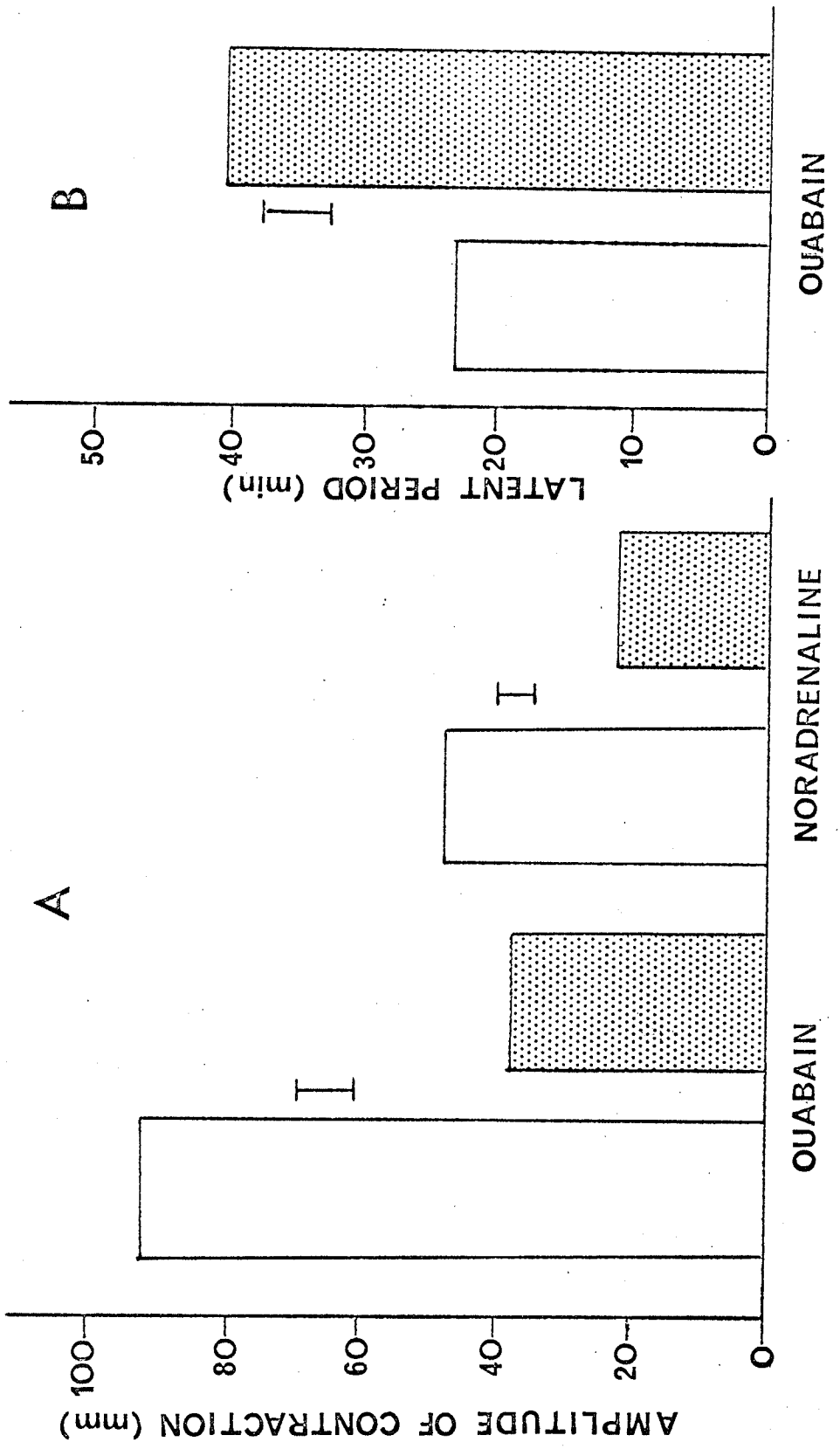

Figure 15. The effect of a decreased extracellular sodium concentration on the response to ouabain. A, amplitude of contractions due to ouabain (3.0 µg/ml) and noradrenaline (1.0 µg/ml). B, ouabain latent period. Open columns, the mean response of strips from four spleens bathed in Krebs-Henseleit solution containing 143 mM sodium. Stippled columns, mean response of strips from the same four spleens bathed in a solution containing 25 mM sodium. Bars between columns show standard errors of the mean difference.

tuted for the NaCl (118 mM). Calcium was omitted from these solutions in order to avoid an irreversible contracture when the strips were immersed in the high potassium medium, and EDTA was added to remove trace amounts of calcium.

After both strips had been in the control medium for sixty minutes, one strip was immersed in the high potassium medium. In every experiment this caused an immediate contraction (mean amplitude 55.3 $\pm$ 11.5 mm), and three to four hours were required for relaxation. Both strips were then tested with noradrenaline (1.0 μg/ml) and tyramine (5.0 μg/ml). On completion of these tests both strips were exposed to ouabain (5.0 μg/ml).

Noradrenaline caused contraction in all strips. The mean amplitudes of contractions for the strips tested in the control and experimental solutions were 15.5 $\pm$ 3.9 mm, and 12.1 $\pm$ 6.0 mm respectively. The difference between the means was not statistically significant. In contrast to noradrenaline, neither tyramine nor ouabain caused contraction in the strips bathed in the high-potassium medium. Both drugs, however, caused contraction in the control strips. The mean amplitude of contraction caused by tyramine was 12.6 $\pm$ 1.8 mm, and that caused by ouabain was 40.0 $\pm$ 12.9 mm. One of these experiments is illustrated in Figure 16.

Two more experiments were carried out to determine whether the effect of the high-potassium medium on the responses to tyramine and ouabain was reversible. The experiments were carried out in much the same way, i.e. one strip remained in the control solution, while the other was bathed in the high-potassium medium for three hours. At the end of this time the control solution was replaced. The strips were left untreated

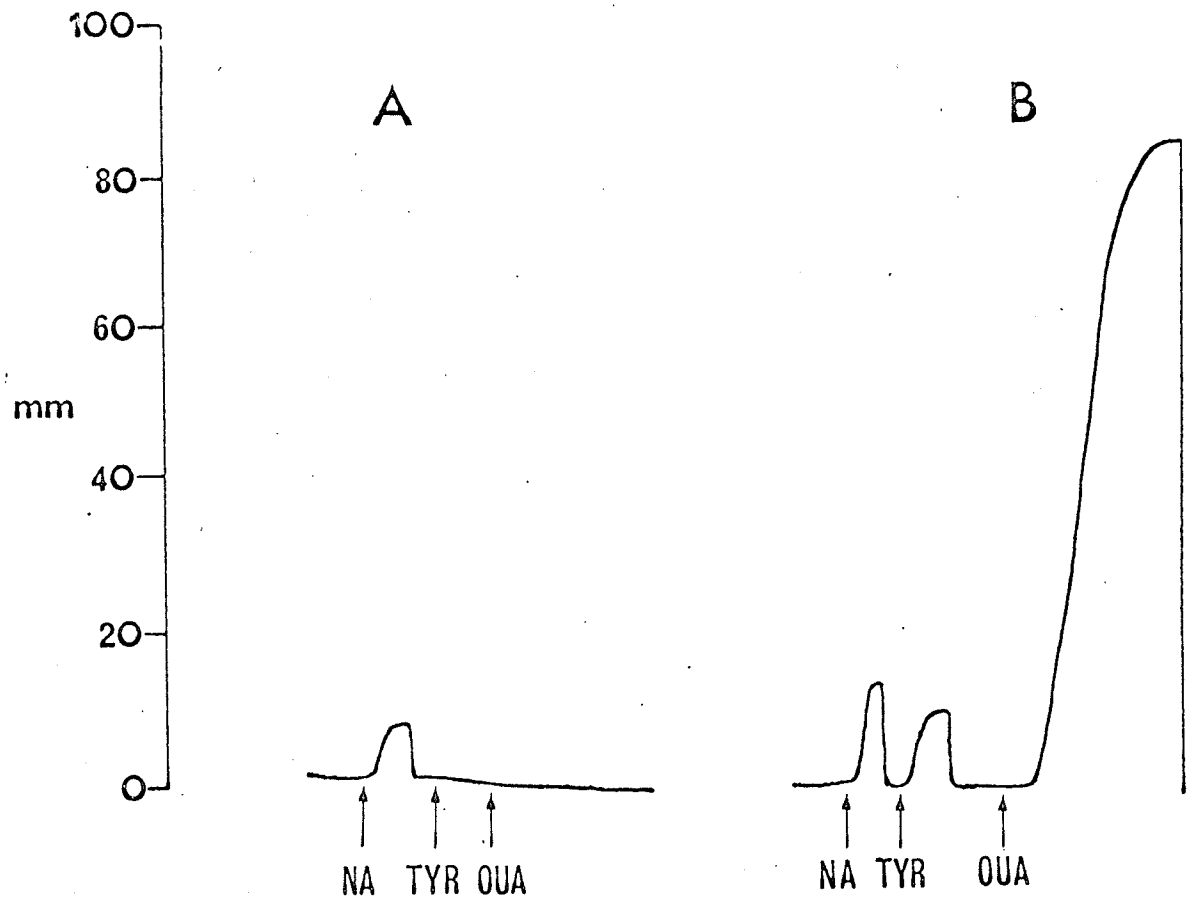

Figure 16. The effect of a high extracellular potassium concentration on the response to ouabain. A, strip bathed in a solution containing 122.7 mM potassium. B, strip from the same spleen bathed in a solution containing 4.7 mM potassium. NA, noradrenaline (1.0 μg/ml), TYR, tyramine (5.0 μg/ml), OUA, ouabain (5.0 μg/ml).

for another sixty minutes; then they were immersed in Krebs-Henseleit solution. After thirty minutes in this solution they were tested with noradrenaline (0.5 μg/ml), tyramine (5.0 μg/ml), and ouabain (5.0 μg/ml).

It was apparent from these experiments that the effect of the high-potassium medium was reversible. In the experimental strips the amplitudes of contractions caused by noradrenaline, tyramine, and ouabain were 5 and 41, 11 and 22, 127 and 126 mm respectively. The corresponding figures in the control strips were 7 and 12, 26 and 30, and 127 and 117 mm.

I. EXPERIMENTS SUGGESTING THAT THE HIGH-POTASSIUM MEDIUM CAUSED RELEASE OF NORADRENALINE.

It was queried whether noradrenaline release contributed to the contraction seen in the above experiments when the strips were immersed in the high-potassium medium. For this reason the contractile effects of the high-potassium medium on four strips from four cats pretreated with reserpine were compared with the effects on four strips from four untreated cats. After sixty minutes in the control solution the strips were immersed in the high-potassium solution.

The change in the bathing medium caused an immediate contraction in all eight strips, but there was an obvious difference between the two groups in both contour and magnitude of the response. The response of the strips from untreated cats consisted of an initial contraction followed by a second phase of slower contraction. In contrast, the response of strips from cats pretreated with reserpine appeared to have only one component and the contraction amplitude was much less (Figure 17). The mean contraction of the control strips was 34.8 $\pm$ 1.9 mm, whereas the corresponding figure for the strips taken from cats pre-

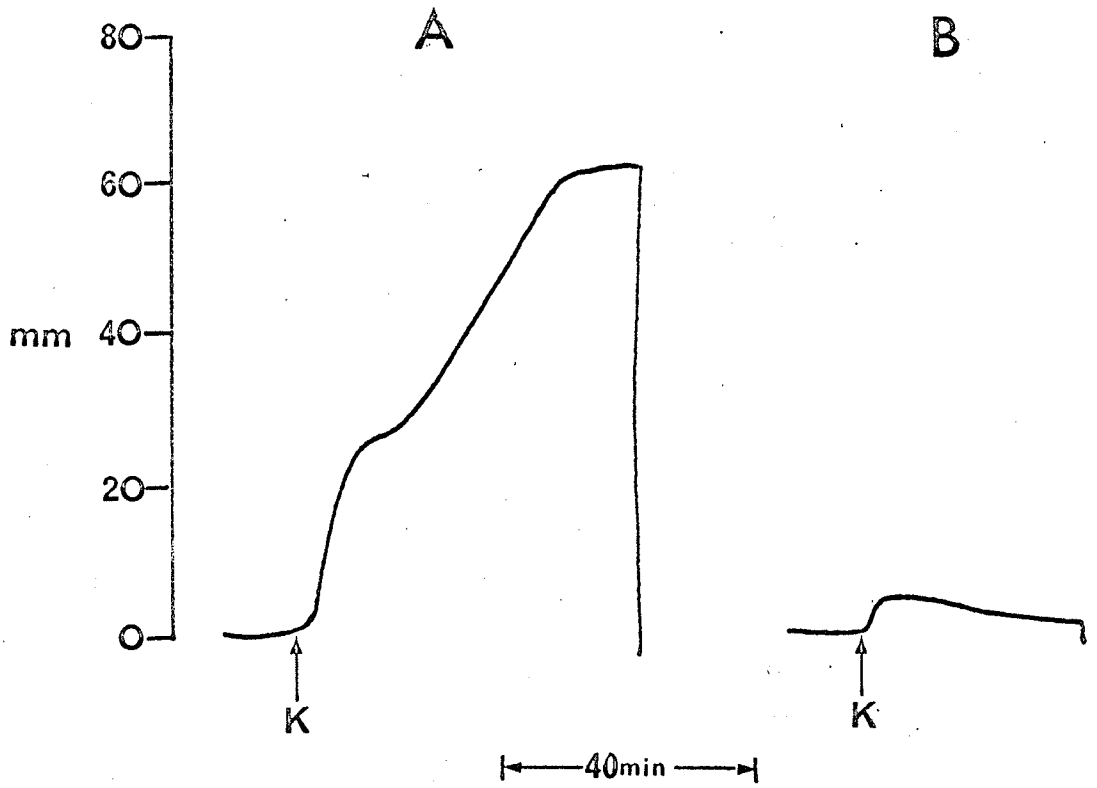

Figure 17. The contractile effect of a high extracellular potassium concentration. A, strip from an untreated cat. B, strip from cats treated with reserpine (1.0 mg/kg) 24 hours before the experiment. K, solution containing 122.7 mM potassium. The mean response for strips from four untreated cats was 34.8 ± 1.9 mm. The mean response for strips from four cats pretreated with reserpine was 11.2 ± 1.9 mm.

treated with reserpine was 11.2 ± 1.9 mm. The difference between the two groups was statistically significant by Student's *t*-test ($P < 0.01$). These experiments suggested that the second component of the response in the control strips was due to the release of noradrenaline.

J. THE EFFECT OF PRONETHALOL ON OUABAIN-INDUCED ARRHYTHMIAS IN RESERPINE-TREATED DOGS.

The finding that pronethalol inhibited the release of noradrenaline from adrenergic fibres caused us to query whether this action was responsible for the antiarrhythmic effect of pronethalol in animals poisoned with digitalis (see Introduction Part II). Even though pronethalol through its β adrenergic blocking action would prevent the chronotropic and inotropic actions of any released catecholamines, there was still the possibility that catecholamines could contribute to arrhythmias through an effect unrelated to activation of β adrenergic receptors.

It was reasoned that if the antiarrhythmic action of pronethalol depended on its ability to inhibit noradrenaline release it should be ineffective against arrhythmias in dogs depleted of noradrenaline. Accordingly, experiments were done on thirteen dogs treated with reserpine (0.5 mg/kg) forty-eight and twenty-four hours before the experiment. This treatment has been shown to deplete the dog heart of all but negligible amounts of catecholamines (Paasonen and Krayer, 1958). Arrhythmias were caused by giving a single intravenous injection of ouabain (85 μg/kg). Ventricular tachycardia developed in each dog within five to seventeen minutes of the injection. Four of the dogs were given no further treatment. Of those, two died thirty-nine and forty-six minutes after receiving ouabain, and ventricular tachycardia persisted unchanged for over three hours in the other two.

Nine dogs were treated with pronethalol fifteen minutes after the arrhythmia began. Five were given a single injection of pronethalol (5 mg/kg), and four were given pronethalol by continuous infusion at a

rate of 200 μg/kg/min. Single injections of pronethalol converted the arrhythmia to sinus rhythm within thirty seconds on each of several (3 to 5) trials in all five dogs (Figure 18). In two of these experiments sinus rhythm was only temporary (1 to 24 min), but in the other three sinus rhythm lasted after the third injection until the experiments were ended one to three hours later. In three of the four dogs treated with infusions the arrhythmias were abolished within three to thirteen minutes, and sinus rhythm was maintained for two hours, after which the infusion was stopped and the experiment ended. In the fourth dog pronethalol (200 μg/kg/min) failed to end the arrhythmia, but after one hour the infusion rate was doubled, and the arrhythmia was immediately abolished. Sinus rhythm was maintained until the experiment was ended one hour later.

The effect of pronethalol on cardiac automaticity in reserpine-pretreated dogs.

The above experiments indicated that the antiarrhythmic effect of pronethalol did not depend on its ability to inhibit the release of noradrenaline from adrenergic nerves. Therefore it was of interest to determine whether the antiarrhythmic effect could be explained by an unspecific depressant effect on cardiac automaticity which was independent of either β receptor blockade or inhibition of noradrenaline release. Accordingly twelve experiments were done on dogs pretreated with reserpine. The normal sinus rate and the frequency of beats originating from a subatrial pacemaker were taken as indexes of cardiac automaticity. Subatrial pacemakers were elicited by stimulating the right vagus (see Methods, page 20).

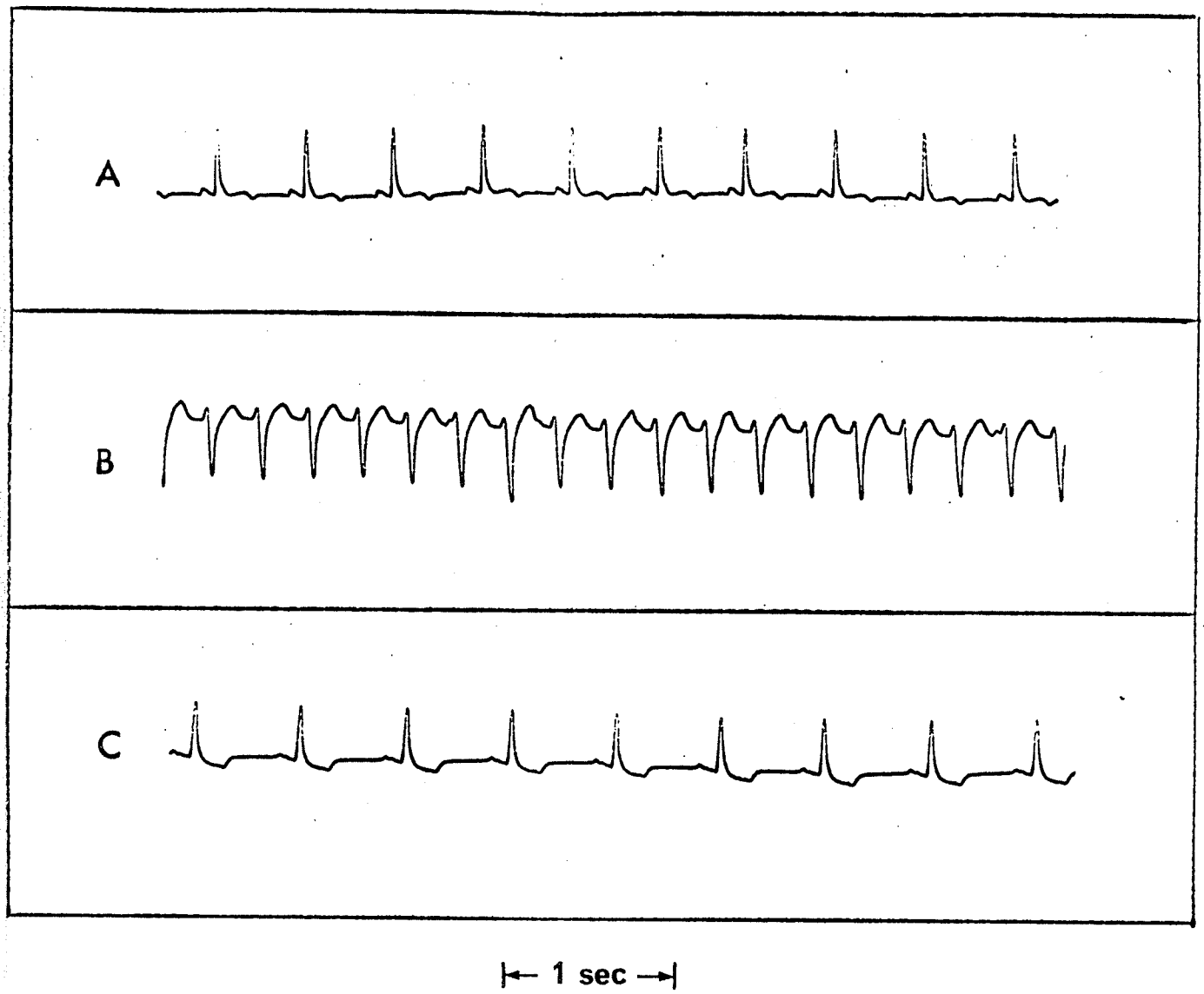

Figure 18. The effect of pronethalol on a ouabain-induced arrhythmia in a reserpine-pretreated dog. Female dog, 18 kg, pentobarbital sodium (20 mg/kg), reserpine (0.5 mg/kg) 48 and 24 hours before the experiment, vagus nerves cut. Lead II ECG. A, sinus rhythm before ouabain. B, arrhythmia after ouabain (85 μg/kg). C, sinus rhythm after pronethalol (5 mg/kg).

In each of four dogs pronethalol (200 μg/kg/min) increased the sinus rate. The average maximum increase in heart rate was 30.2 ± 5.2 beats/min and occurred within three to nine minutes after the start of the infusion. The heart rate remained above the pre-infusion level throughout the period of infusion (30 to 45 min) (Figure 19).

In four other dogs the frequency of the subatrial rhythm during vagal escape was measured before and ten minutes after the beginning of the pronethalol (200 μg/kg/min) infusion. A period of ten minutes of infusion was chosen because this was about the time required in the earlier experiments for a similar infusion of pronethalol to reverse ouabain-induced arrhythmias. In all four dogs pronethalol increased the frequency of beats without restoring sinus rhythm (Figure 20). The average increase was 14.5 ± 1.2 beats/min. This increase was statistically significant by paired data ($P < 0.01$). In another four dogs pronethalol was withheld and two measurements of the subatrial rate were made ten minutes apart. There was no significant increase between the first and second measurement (+ 1.25 ± 1.3 beats/min). The difference between the dogs given pronethalol and the control group was statistically significant ($P < 0.01$).

Since pronethalol increased cardiac automaticity in these experiments, the antiarrhythmic effect could not be explained by an unspecific depression of automaticity. However, the finding that pronethalol increased the sinus rate of dogs pretreated with reserpine suggested that the agent might have reversed the arrhythmias by accelerating the rate of the sinus pacemaker above that of the ectopic foci. If this were the case, the sinus rate after conversion of the arrhythmia should be faster than the ventricular rate during the arrhythmia. The

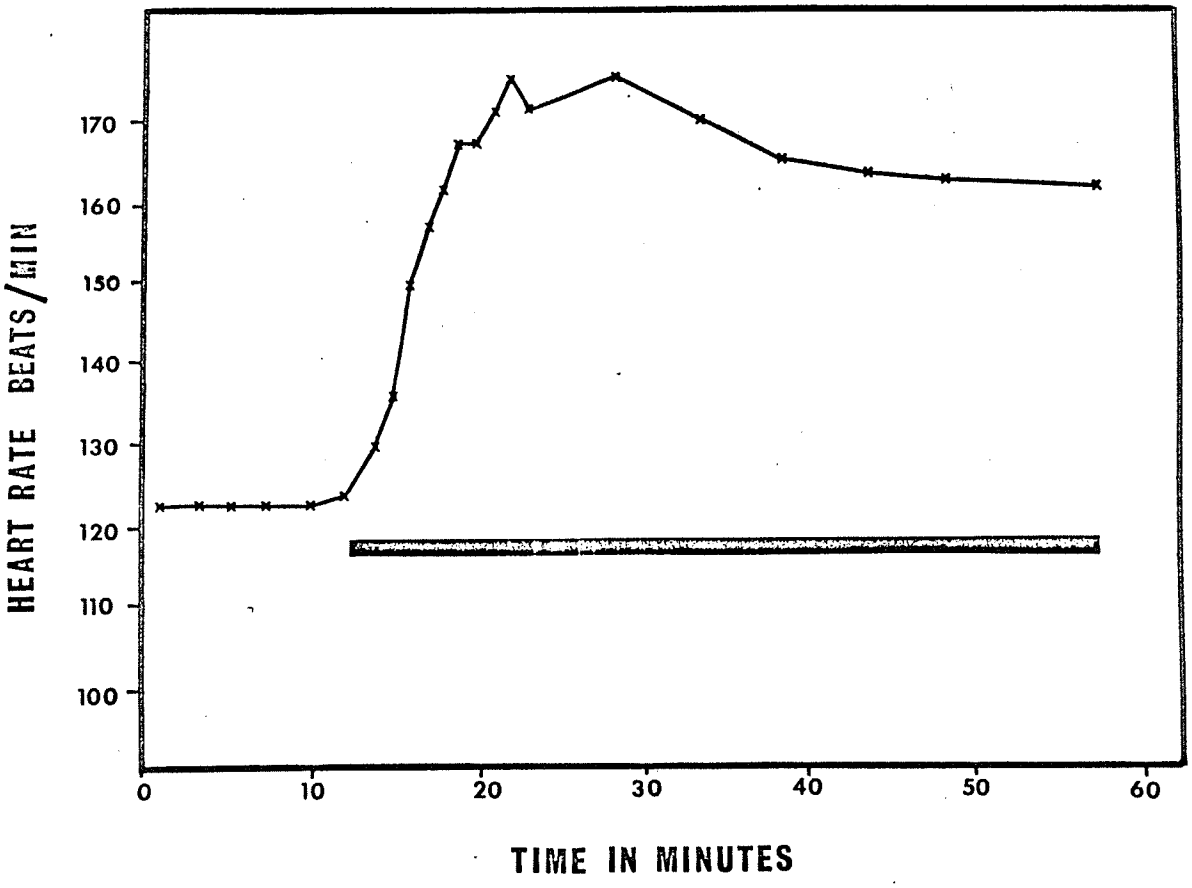

Figure 19. The effect of pronethalol on the heart rate of a reserpine-pretreated dog. Male dog, 15 kg, pentobarbital sodium (20 mg/kg), reserpine (0.5 mg/kg) 48 and 24 hours before the experiment, vagus nerves cut. Bar indicates pronethalol (200 μg/kg/min) infusion. The mean increase in heart rate for four such animals was 30.2 ± 5.2 beats/min.

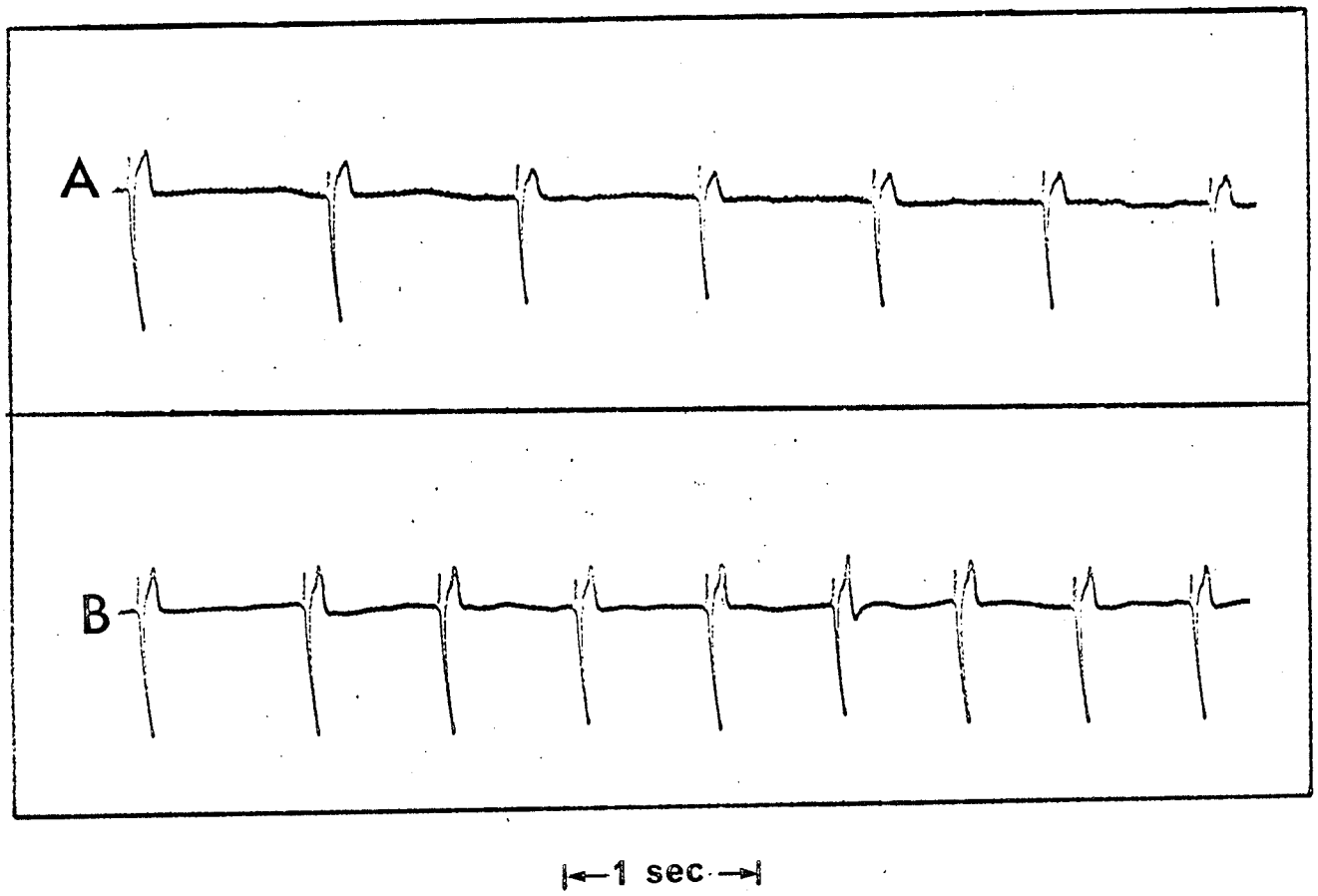

Figure 20. The effect of pronethalol on the rate of a subatrial pacemaker in a reserpine-pretreated dog. Female dog, 10 kg, pentobarbital sodium (20 mg/kg), reserpine (0.5 mg/kg) 48 and 24 hours before the experiment, vagus nerves cut. Subatrial pacemaker elicited by stimulation of the peripheral end of the cut right vagus (twice threshold voltage, 20 pulses/second, 1.0 millisecond duration). Lead II ECG. A, before pronethalol; rate 60 beats/minute. B, ten minutes after start of pronethalol (200 μg/kg/min) infusion; rate 83 beats/minute.

ventricular rates that existed just before abolishing the arrhythmias were therefore compared with the sinus rates that existed just after abolition of the arrhythmia. Conversion of the arrhythmia always slowed the rate. In dogs treated with single injections of pronethalol the mean rate of the ventricular pacemakers before treatment was 198 $\pm$ 6.5 beats/min; the subsequent sinus rate was 169 $\pm$ 3.7 beats/min. The decrease was statistically significant ($P < 0.01$). In the dogs treated with pronethalol infusions the mean rate of the ventricular pacemakers before treatment was 189 $\pm$ 6.2 beats/min, while the subsequent sinus rate was 143 $\pm$ 4.3 beats/min. Again the decrease was statistically significant ($P < 0.02$).

DISCUSSION

The results clearly show that ouabain can release noradrenaline. There is no other single explanation for the experiments showing that the ouabain-induced contraction of the spleen was all but abolished by the α adrenergic blocking agent phenoxybenzamine, or by depletion of noradrenaline by either pretreatment with reserpine or chronic denervation of the spleen. In addition, this result of chronic denervation established that the entire source of the released noradrenaline was the postganglionic sympathetic fibres.

In addition to its releasing action ouabain was shown to have a contractile effect on the spleen which was not due to release, i.e. it caused contraction in spleen strips depleted of noradrenaline when extremely high concentrations were used. The concentration of ouabain required for a maximal contraction in depleted strips was over 150 times greater than that necessary to elicit a maximal contraction in strips containing noradrenaline. This wide separation in effective concentrations made the spleen strip an ideal preparation for this study because we could be sure that any significant contraction occurring with concentrations of less than 25.0 µg/ml was due to the release of noradrenaline.

It is most probable that the contraction caused by ouabain in strips from cats pretreated with reserpine was due to a direct action on the splenic smooth muscle. The contractile effects of digitalis on smooth muscle have been known for many years. Prior to 1925 it had been shown on many *in vitro* preparations, e.g. frog and mammalian vessels; frog and toad esophagus; mammalian stomach, gut, non-pregnant uterus, and vas deferens. *In vivo*, toxic doses of cardiac glycosides were observed to have contractile effects on mammalian vessels, bronchi, gut, spleen, and pregnant uterus (see Cushny, 1925). These early experiments were not

designed to show whether the observed effects were due to a direct action or to the release of an active endogenous substance. However, many of the experiments were carried out in the presence of atropine, which ruled out acetylcholine, and the contractile effects in the bronchi and non-pregnant uterus could not be explained by the release of noradrenaline since noradrenaline relaxes rather than contracts these tissues.

In more recent years it has been well established that the cardiac glycosides do have a direct contractile action on smooth muscle. The work of Daniel (1964a, 1964b) provides strong evidence which indicates that the action is due to the downhill ion movement caused by the glycosides (see Introduction Part IV). In his view downhill ion movement promotes the entry of calcium into the smooth muscle cells and initiates contraction.

Protection of noradrenaline receptors against phenoxybenzamine blockade by ouabain.

The protection technique was originated by Furchgott (1954) as a method for identifying specific drug receptors. In theory, when a drug is present in the bathing medium in high concentrations it occupies a large proportion of its receptors and thus limits the access of the blocking agent to these receptors but not to other receptors. For example, Furchgott concluded that the rabbit aorta had specific receptors for acetylcholine, histamine, 5-hydroxytryptamine, and noradrenaline because each agonist protected only itself against blockade. On the other hand, he concluded that noradrenaline, adrenaline, and isoproterenol acted on the same receptors because any one of the three would protect not only itself but the other two as well.

We showed that ouabain protected noradrenaline but not histamine receptors in spleen strips from normal cats (pages 42-46). However, in strips depleted of noradrenaline by pretreatment of the cats with reserpine, ouabain failed to protect either noradrenaline or histamine receptors. This strongly suggested that the protection afforded by ouabain in the strips from normal cats was due to the release of endogenous noradrenaline. This finding, the first demonstration of protection by endogenous noradrenaline, suggests that the amount of noradrenaline released by ouabain from the spleen is considerable.

In addition our experiments point out the necessity of several controls when employing the protection technique. For instance, in the strips from reserpine-treated cats the remaining response to noradrenaline was slightly, but significantly greater in the strips exposed to both ouabain and phenoxybenzamine than it was in the strips exposed to only phenoxybenzamine. However, it would have been erroneous to conclude that this was due to protection, since the response to noradrenaline was significantly potentiated in the strips exposed to ouabain alone. The dose-response curves (pages 47-48) showed that the potentiation of noradrenaline by ouabain occurred over a wide range of noradrenaline concentrations. Moreover, the potentiation appeared to be specific since ouabain failed to potentiate histamine in the protection experiments.

No attempt was made to determine the mechanism involved in the potentiation of noradrenaline by either ouabain or pronethalol (pages 49-50). However, it is believed by some that potentiation by certain drugs such as cocaine results from blockade of uptake sites, i.e. sites other than the active receptors which lead to the response (for reviews see

Trendelenburg, 1963, 1966). According to this theory blocking these sites diverts some of the agonist which would normally have been taken up, so that the concentration of agonist available for combination with the active receptors is increased. The experimental basis for the theory is that cocaine blocks the uptake of catecholamines (Muscholl, 1961). More recently it has been shown that ouabain blocks uptake of noradrenaline in isolated slices of cat heart and brain (Dengler, 1964), and pronethalol has been shown to block noradrenaline uptake in the isolated perfused rabbit heart (Lindmar and Muscholl, 1964). Therefore the "uptake" theory may apply to the potentiation produced by ouabain and pronethalol as well as to that caused by cocaine.

The loss of tissue potassium.

Section E showed that ouabain caused a loss of potassium from the spleen strip. This indicated that downhill ion movement had taken place with its attendant decrease in intracellular potassium and gain in sodium, and for the reasons already pointed out (Introduction Part IV) the primary event must have been the inhibition of transport ATPase. However, the purpose of these experiments was not to show simply that ouabain caused downhill ion movement in the spleen, but to determine whether this could be related to the release of noradrenaline caused by ouabain. Our evidence, although circumstantial, suggests that it can. There is, however, a discrepancy that must first be explained before proceeding with the main arguments.

The loss of potassium and the release of noradrenaline, as judged by the onset of contraction, were not closely correlated in time. Maximal potassium loss occurred within fifteen minutes of adding ouabain

to the bath, but the contraction did not start until several minutes later. This is not surprising since the entire source of noradrenaline was the adrenergic nerve fibres, whereas any of the cell types in the spleen were potentially susceptible to potassium loss (see page 14-15). Therefore, ionic changes occurring in the nerve fibres would have been masked by the changes occurring in other cells. Nevertheless, a causal relationship between downhill ion movement and noradrenaline release was suggested by the action of pronethalol. It delayed both downhill ion movement and the release of noradrenaline, as well as reducing the amount of noradrenaline released. Since pronethalol obviously opposed downhill ion movement in some cells, it is reasonable to assume that it also opposed downhill ion movement in the adrenergic fibres.

The differing time course between the measured loss in potassium and the onset of contraction implies that at least some of the cells in the spleen are more susceptible to the action of ouabain than are the adrenergic nerve fibres. This point is easily accepted, for it is well known that different cell types vary widely in their sensitivity to digitalis-induced downhill ion movement (Glynn, 1964), and it has been shown that nerve is less sensitive than most other tissues (Skou, 1957).

General characteristics of the ouabain-induced contraction.

The view that downhill ion movement is responsible for the release of noradrenaline is compatible with the general characteristics of the ouabain-induced contraction. The long latent period between the addition of ouabain to the bath and the onset of contraction is consistent with the work of Glynn (1957) and others (for review see Glynn, 1964) who showed that the inhibition of active transport by cardiac glycosides is

slow to develop. Glynn (1957) showed that when red cells were exposed to a small concentration of glycoside the inhibition continued to increase over a three hour period. However, when he used a high concentration of glycoside maximum inhibition was reached within a few minutes. This observation fits well with our data showing that the latent period was inversely related to the concentration of ouabain (pages 31-32).

We found that spleen strips exposed to ouabain (3 μg/ml) for less than twenty minutes failed to contract. This indicated that the uptake of ouabain by the tissue from the bathing fluid was slow. This agreed with an observation of Glynn (1957) which showed that the uptake of scillaren A by red cells was slow. Glynn suggested that this slow uptake was responsible for the latent period between the addition of the glycoside to the bathing medium of the red cells and the inhibition of active transport. Moreover, Glynn was able to correlate the duration of exposure of the red cells to scillaren A with the degree of inhibition of active transport, i.e. the longer the duration of exposure the greater the inhibition of the active accumulation of potassium. Similarly, we found some correlation between the amount of noradrenaline released (as judged by the amplitude of contraction) and the duration of exposure of the spleen strip to ouabain (pages 28-29).

Glynn's data also suggested an answer as to why we failed to relate the amplitude of contraction to the concentration of ouabain. They showed that the digitoxin-induced inhibition of potassium accumulation by the red cells measured after one hour increased sharply with a very small increase in glycoside concentration. The dose-response curve rose very steeply between 0.01 and 0.1 μg/ml of digitoxin, and only about

a twenty per cent increase in inhibition could be gained by increasing the concentration from 0.1 to 10.0 μg/ml. These data in combination with ours suggest that it might have been possible to obtain graded responses to ouabain in the spleen strip by using very closely spaced increments in concentration between 0.1 and 1.0 μg/ml. This, however, was not attempted because it was thought that the additional data would have been of little use in elucidating the mechanism of the ouabain-induced contraction.

The effect of an increased extracellular calcium concentration.

The effect of an increased extracellular calcium concentration on excitable cells, e.g. smooth muscle, nerve, striated muscle, and Purkinje fibre has been reviewed by Shanes (1958), and more recently by Bohr (1964) and Schatzmann (1964). These authors point out that the downhill passage of sodium into the cell both at rest and during depolarization is reduced when the extracellular calcium concentration is elevated. It follows from what was said earlier in the Introduction (page 16) about the inseparability of sodium and potassium movements, that the downhill movement of potassium out of the cell is also reduced. It is this action of calcium that forms the basis of its membrane stabilizing effect (Schatzmann (1964).

We found that increasing the calcium concentration of the Krebs-Henseleit solution from 2.5 to 5.0 mM significantly increased the latent period and reduced the height of the ouabain-induced contraction (pages 54-55). The smaller contraction could not be explained by a decreased responsiveness of the tissue to noradrenaline because the contractions elicited by exogenous noradrenaline were enhanced. Therefore these experiments indicated that the noradrenaline releasing action of ouabain

was antagonized by increasing the calcium concentration. Thus these experiments further implicated downhill ion movement in the release of noradrenaline.

The effect of a sodium deficient medium.

The effect of reducing the sodium concentration of the bathing fluid is similar to increasing the calcium concentration in that both procedures increase membrane resistance (for review see Shanes, 1958). We found that decreasing the sodium concentration (from 143 to 25 mM) was similar to increasing the calcium concentration in that it reduced the amplitude and increased the latent period of the ouabain-induced contraction (pages 55&57). This was consistent with the idea that downhill ion movement was responsible for the release of noradrenaline by ouabain, because the amount of sodium available to run downhill was reduced, and the membrane resistance to the passage of ions was probably increased.

However, definite conclusions cannot be drawn from these experiments because there were several complicating factors. The smaller contraction due to ouabain could not be taken as evidence that less noradrenaline was released, because the sensitivity of the tissue to exogenous noradrenaline was impaired by the low sodium solution. On the other hand the increased latent period suggested that the time required for ouabain to release noradrenaline was increased, for it seems unlikely that the impaired sensitivity of the tissue to noradrenaline could account for an increase in the latent period of seventeen minutes.

Further complications arise from the fact that the sodium concentration was reduced by substituting sucrose for the sodium chloride of the Krebs-Henseleit solution. Therefore we do not know whether the

removal of chloride contributed to the antagonized response to ouabain, nor do we know whether sucrose itself had an effect. Obviously, sodium substitutes besides sucrose must be tried in the near future. It will also be of interest to determine whether the release of noradrenaline by ouabain can be abolished by the entire removal of sodium from the bathing medium.

The effect of depolarization by potassium.

It is known that smooth muscle still responds to many agonists even when the sodium chloride of the bathing fluid has been replaced by potassium chloride so that the muscles are depolarized (Evans, Schild and Thesleff, 1958; for reviews see Daniel, 1964b and Schild, 1964). When we replaced the sodium chloride of Krebs-Henseleit solution with potassium chloride (potassium-Krebs), it was necessary to leave out the calcium; otherwise an irreversible contracture occurred in the spleen strip. Consequently we were obliged to omit the calcium from our control solution (solution in which the sodium chloride was not replaced, sodium-Krebs).

The results showed that removal of calcium from sodium-Krebs did not prevent contractions caused by noradrenaline (pages 59-61). This applied to noradrenaline released from the tissue by either tyramine or ouabain as well as to exogenous noradrenaline added to the bath. This observation was expected, for it is known that smooth muscle contractions elicited by noradrenaline are relatively insensitive to the absence of extracellular calcium. For example, Hinke (1965) has shown in isolated vascular smooth muscle that potassium-induced contractions quickly decay after removal of calcium from the bathing medium, but that contractions

caused by noradrenaline do not. It is thought that noradrenaline acts on a tightly bound store of calcium which is not readily depleted by removing extracellular calcium; whereas potassium-induced contractions rely on a loosely bound store that is susceptible to removal of extracellular calcium (Hinke, 1965).

Although removal of calcium from the bathing fluid did not prevent contractions caused by tyramine and ouabain, substitution of potassium chloride for sodium chloride did. Abolition of the responses could not be explained on the basis of unspecific damage to the tissue, because noradrenaline added to the bath was as effective in causing contraction in the substituted solution as it was in the control solution. Moreover, responsiveness to tyramine and ouabain could be restored by returning the tissues to Krebs-Henseleit solution. Thus, the results indicated that the high potassium concentration interfered with the ability of tyramine and ouabain to release noradrenaline.

The failure of ouabain to act in potassium-Krebs was not surprising. It has already been pointed out in the Introduction that increasing the extracellular potassium concentration decreases the inhibiting effect of cardiac glycosides on transport ATPase (page 15). In addition, depolarization by potassium greatly increases the permeability of the cell to sodium and potassium (Shanes, 1958), and under these conditions a net downhill ion movement is favored. Therefore, downhill ion movement was probably already present at the time ouabain was added and consequently ouabain could cause little further change. These points are consistent with the general hypothesis that the ouabain-induced release of noradrenaline is due to inhibition of the active sodium-potassium

transport and its consequent downhill ion movement.

Observations suggesting the release of noradrenaline by potassium-Krebs.

Even though the spleen strips were in calcium-free sodium-Krebs for sixty minutes, contractions occurred when this solution was replaced with calcium-free potassium-Krebs. This suggested the possibility that noradrenaline was released when the solutions were changed, for potassium-induced contractions are known to be more sensitive to the absence of extracellular calcium than contractions caused by noradrenaline. This suggestion was reinforced when we compared the contractions caused by potassium-Krebs in strips taken from normal cats and in strips from cats pretreated with reserpine. In the strips from cats without reserpine the contractions consisted of two components, whereas in the strips from reserpine-pretreated cats the contractions were much smaller and consisted of only one component (page 61). This suggested that noradrenaline was responsible for part of the contraction.

The failure of tyramine to act in potassium-Krebs.

The inability of tyramine to release noradrenaline from tissues depolarized by potassium was an unexpected and original observation (pages 59-60). The data suggesting that the bathing medium (potassium-Krebs) released noradrenaline provide a possible explanation. The store of noradrenaline which is available to tyramine may have been exhausted by the time tyramine was added. If this was the case the store must have been replenished when the tissue was returned to Krebs-Henseleit solution since the response to tyramine was then restored. It will be of interest in future experiments to determine whether the releasing action of other

indirectly acting sympathomimetics is prevented by depolarizing solutions.

Initiation of the ouabain-induced contraction by exogenous noradrenaline.

Our finding that exogenous noradrenaline hastened the response to ouabain (pages 31-35) requires further experiments before any conclusions regarding its mechanism can be made. However, the failure of histamine to have a similar effect showed that more than the contraction caused by exogenous noradrenaline was involved. Conn and Luchi (1964) have reported that adrenaline and noradrenaline increase the permeability of cardiac cells to sodium and bring about an increase in cell sodium with a small loss of potassium. A similar effect on adrenergic fibres would be expected to augment the downhill ion movement caused by ouabain, and thus perhaps facilitate the release of noradrenaline. Unfortunately, whether noradrenaline has this effect on adrenergic fibres is not known.

Calcium as a link in the release of catecholamines.

A pattern of circumstantial evidence has been presented which strongly suggests that inhibition of active sodium-potassium transport and its consequent downhill ion movement are responsible for the ouabain-induced release of noradrenaline. There is, however, no way of knowing from our data whether some step lies between downhill ion movement and the release of noradrenaline. For example, it is known that during depolarization the influx of sodium into nerve is accompanied by an influx of calcium (Hodgkin and Keynes, 1957), and, as already mentioned, Daniel (1964a, 1964b) believes that the direct contractile effect of cardiac glycosides on smooth muscle is due to downhill ion movement which promotes the entry of calcium into the cells.

Most pertinent, though, is the work of Douglas and co-workers (1961, 1962, 1963, 1965, and 1966), which has shown that entry of calcium into the chromaffin cells of the adrenal medulla causes release of catecholamines. They have also shown that calcium is the only ion essential in the release of catecholamines from the medulla by either acetylcholine or potassium. In their view the role of calcium in this regard is analogous to its role in excitation-contraction coupling in muscle, and they have coined the term "stimulus-secretion coupling". This work, therefore, raises the question, is the ouabain-induced release of noradrenaline from the spleen due only to entrance of calcium into the adrenergic fibres?

Our data do not permit a definite answer to this question, but they suggest that the answer is no. The first indication was from the experiments showing that an increase in extracellular calcium concentration from 2.5 to 5.0 mM inhibited the release of noradrenaline by ouabain. A longer time for the release to occur was required, and it appeared from the reduced contraction that less noradrenaline was released. This is in sharp contrast to the work of Douglas (1965) who found that increasing the calcium concentration from 2.2 to 8.8 mM greatly enhanced the amount of catecholamine released by acetylcholine from the adrenal medulla. Moreover, Douglas and Rubin (1961) clearly showed that neither acetylcholine nor excess potassium release catecholamines if calcium is absent from the perfusing medium. Yet we found that either ouabain or excess potassium released noradrenaline when calcium was absent from the bathing medium and a high concentration of EDTA was present. Our experiments carried out in the sodium deficient medium also bear on this question. The work of others has established that lowering the extracellular

sodium concentration facilitates entry of calcium into muscle cells (Shanes, 1963). Consistent with this is the observation of Douglas and Rubin (1963) that lowering the sodium concentration greatly increased the amount of catecholamine released from the adrenal medulla. However, we found that reducing the extracellular sodium concentration opposed the release of noradrenaline by ouabain.

The above points suggest that the fundamental mechanisms involved in the release of catecholamines from the chromaffin cells of the adrenal medulla and the release of noradrenaline from adrenergic fibres in the spleen are different. On the other hand, Burn and Gibbons (1965) think that calcium is involved in the release of noradrenaline from adrenergic fibres in the same way as it is in the adrenal medulla. Their work, however, is not nearly so convincing as Douglas's. Fundamental to their work was the finding that the inhibition of the isolated rabbit ileum due to electrical stimulation of the peri-arterial nerves was enhanced by increasing the calcium concentration of the bathing medium. They concluded that the enhancement was due to an increased release of noradrenaline. However, they ignored the earlier work of Bülbring and Kuriyama (1963) showing that an increase in extracellular calcium concentration markedly potentiated the inhibiting effect of exogenous adrenaline on the guinea-pig taenia coli. Indeed, Bueding and Bülbring (1964) have advanced the hypothesis that the smooth muscle inhibitory effect of adrenergic drugs is due to membrane stabilization resulting from an increase of calcium at the cell membrane. In contrast to Bülbring and Kuriyama, Burn and Gibbons (1964) reported that elevating the calcium concentration had little effect on the inhibitory effect of

exogenous noradrenaline in rabbit ileum, but their data reveal that only one concentration of noradrenaline (0.01 μg/ml) was tried, and even this was potentiated in some experiments by an increased extracellular calcium.

Burn and Gibbons (1965) also reported that calcium promotes the release of noradrenaline from adrenergic fibres in the isolated rabbit atria. Noradrenaline release was elicited by either nicotine or acetylcholine, and the depressant cholinergic effect of these drugs was blocked by hyoscine. They found that elevating the calcium concentration consistently enhanced the stimulating effect of acetylcholine and nicotine on this preparation, although its effect on exogenous noradrenaline was inconsistent.

The importance of noradrenaline release in the therapeutic action of digitalis on the heart.

The recent experiments of Loubatières, Bouyard, Chapal, Klein, and Rondot (1965) provide direct evidence that ouabain can cause the release of noradrenaline from the heart. Six hours after injecting guinea-pigs with 125 μg/kg of ouabain there was a marked reduction in chemically assayed cardiac catecholamines. The loss in total amine was accounted for almost entirely by noradrenaline; there was only a very slight reduction in adrenaline. These results combined with ours lend strong support to the original suggestion of Tanz (1960) that ouabain could cause the release of catecholamines from the heart.

It is not unlikely that ouabain acts similarly on adrenergic fibres in the heart and spleen. Certainly other drugs do, e.g. tyramine and reserpine. In this regard, it is of interest to compare the concentration of ouabain required to release noradrenaline from the spleen to

the concentration used by Tanz to demonstrate a reduced inotropic response after reserpine or DCI (see Introduction). The minimum concentration that consistently caused contraction of the spleen was 0.3 μg/ml, which is very close to the concentration, 0.5 μg/ml, used by Tanz. Moreover, there is a similarity in the time course in our results on the spleen and those of Tanz (1964) on the cat papillary muscle. About forty-five minutes were required for 0.5 μg/ml to cause a significant release of noradrenaline from the spleen, and Tanz's records show that about the same time was required for this concentration to exert its peak inotropic effect.

Although our work on the spleen established that ouabain was capable of releasing noradrenaline from adrenergic fibres, it also showed the improbability of this being the important factor in the therapeutic action of digitalis. The concentrations of ouabain necessary to cause release were clearly not therapeutic. It has been known since the classical work of Cattell and Gold in 1938 that concentrations of glycoside much smaller than those we found necessary to release noradrenaline exert a therapeutic effect on the heart. Also, the data of Tanz show that a toxic concentration is needed to demonstrate an adrenergic component in the inotropic response to ouabain. In most of his studies beating of the preparation was arrested in slightly over two hours. The dose of ouabain used by Loubatières *et al.* to reduce the myocardial content of catecholamines is highly toxic. As already discussed our results indicated that splenic adrenergic fibres were more sensitive to ouabain than splenic smooth muscle. In contrast to the spleen, it would appear in the heart that cardiac muscle is more sensitive to ouabain than cardiac adrenergic fibres, and it is this fact which precludes noradrenaline re-

lease as a significant factor in the therapeutic action of digitalis.

It is not surprising that so much controversy exists in the literature on this subject (see Introduction). For the reasons just pointed out it is unlikely that any investigator who used a therapeutic dose of digitalis would find evidence of catecholamine release. Even when large doses are used there is the possibility that the adrenergic component could be masked. For example it has been shown by Mendez, Aceves, and Mendez (1961) as well as others (Nadeau and James, 1963; Tuttle and Innes, 1966) that cardiac glycosides inhibit the chronotropic action of catecholamines, and Godfraind and Godfraind-De Becker (1964) found that the inotropic action was also antagonized. A recent study by Förster and Kalsow (1965) indicates that the possibility of demonstrating an adrenergic component may depend on which glycoside is used. They found that DCI antagonized the positive inotropic effect of digitoxigenin, bulfalin, and ouabain, but the effect of strophanthidin, digoxigenin, and digoxin was equivocal.

The effect of pronethalol on digitalis-induced arrhythmias.

The antagonism of digitalis-induced arrhythmias by pronethalol is due neither to prevention of release nor to blockade of catecholamines. This conclusion is based on our experiments showing that pronethalol converted arrhythmias caused by ouabain to sinus rhythm in dogs depleted of catecholamines by pretreatment with reserpine (pages 64-66). Clearly, pronethalol could not affect catecholamines that were not present. The work of Lucchesi (1964, 1965) and Somani and Lum (1965) provides further evidence that β receptor blockade is inadequate to explain the antiarrhythmic effect of pronethalol (see Introduction pages 8-10).

Somani and Lum have suggested that the antiarrhythmic effect of pronethalol is due to an unspecific "quinidine-like" action. Since quinidine has many actions it would not be surprising to discover that it shares a common property with pronethalol. However, our results showed that there was an important difference between these two drugs, and for this reason I believe that the term "quinidine-like" is more misleading than informative.

It is generally accepted that the principal direct action of "quinidine-like" drugs on the heart is depression of all functions including automaticity. Accordingly, if the antiarrhythmic effect of pronethalol relies on a "quinidine-like" action, the drug should depress cardiac automaticity in antiarrhythmic concentrations. Such an action would oppose the increased ventricular automaticity caused by toxic doses of cardiac glycosides, and thus tend to suppress the arrhythmia. This explanation however, is not supported by our results. In dogs depleted of catecholamines pronethalol stimulated rather than depressed automaticity. It caused an increase in the normal sinus rate and in the frequency of beats originating from subatrial pacemakers. These results are not surprising, for pronethalol has been previously shown to possess intrinsic sympathomimetic activity (Sekiya and Vaughan Williams, 1963; Donald, Kvale and Shepherd, 1964). The significant point is that the dose of pronethalol used in these animals was the same as that used to reverse ouabain-induced arrhythmias in other animals similarly depleted of catecholamines. This indicates that pronethalol can exert its antiarrhythmic effect in a concentration which tends to stimulate rather than depress automaticity. This is a property not shared by "quinidine-like" drugs. Another dissimilarity between the antiarrhythmic action of pro-

nethalol and quinidine is apparent from the work of Somani and Lum (1965), who found that pronethalol had no influence on arrhythmias caused by coronary ligation. In contrast, quinidine is effective against arrhythmias caused by this method (Harris, Estandia, Ford and Tillotson, 1951; Winbury and Hemmer, 1955; Clark and Cummings, 1956).

The basis of pronethalol's antiarrhythmic action.

The early work of Calhoun and Harrison (1931) established that arrhythmias caused by cardiac glycosides are associated with loss of myocardial potassium. Since then it has become generally accepted that downhill ion movement is the cause of digitalis-induced arrhythmias (Regan, London, Binak and Hellems, 1962; Conn and Luchi, 1964; Luchi and Conn, 1965). Our results from the spleen strip showed that pronethalol opposed the downhill ion movement caused by ouabain. El-Fiky and Katzung (1965) concluded that this effect of pronethalol is basic in its antiarrhythmic action since it prevented both the cardiac downhill ion movement and arrhythmias caused by toxic doses of ouabain in dogs.

SUMMARY

1. Ouabain in concentrations of 0.3 μg/ml or more caused a marked contraction of isolated cat spleen strips. The contraction was sustained, requiring two to four hours for relaxation. After relaxation, the contraction to a second dose of ouabain was greatly reduced. There was a long latent period (4 to 120 minutes) between the addition of ouabain to the bath and the onset of contraction. The contraction height and latent period among strips from the same spleen and exposed to the same concentration of ouabain were similar, but there was much more variability among strips from different spleens. The contraction height was not correlated with the concentration of ouabain, but the latent period was inversely related to concentration, i.e. the higher the concentration the shorter the latent period.

2. The entry of ouabain into the tissue appeared to be slow. If ouabain (3.0 μg/ml) was washed out of the bath in less than twenty minutes the contraction did not occur. The duration of exposure appeared to be related to the height of contraction, i.e. the longer the exposure the greater the contraction.

3. The latent period was shortened by noradrenaline, added to the bath after ouabain but before the contraction due to ouabain usually occurred. The shortening of latent period was not due to contraction because contractions caused by histamine failed to alter the latent period.

4. Three types of experiments indicated that the ouabain-induced contraction was due to the release of noradrenaline. The contraction was nearly abolished by the α adrenergic blocking agent phenoxybenzamine. Depletion of noradrenaline by treatment of the cats with reserpine reduced contractions due to ouabain to less than six per cent of that of strips from untreated cats. Ouabain failed to cause contraction

in strips from spleens depleted of noradrenaline by chronic denervation. Strips depleted of noradrenaline by either reserpine or chronic denervation were more responsive to exogenous noradrenaline than control strips.

5. In addition to releasing noradrenaline ouabain appeared to have a direct action on splenic smooth muscle. In very high concentrations ouabain caused contraction of spleen strips from cats treated with reserpine. The concentration needed to cause contraction in depleted strips was about 150 times greater than that needed to cause contraction in strips containing noradrenaline.

6. Ouabain protected noradrenaline but not histamine receptors from blockade by phenoxybenzamine. It was concluded that protection was due to noradrenaline release since ouabain failed to protect in strips from cats treated with reserpine.

7. In strips from cats treated with reserpine, ouabain potentiated the responses to exogenous noradrenaline. Since ouabain is known to block uptake of noradrenaline this was suggested as possible explanation for the potentiation.

8. Pronethalol reduced the height of the ouabain-induced contraction. It was concluded that this was due to a decreased release of noradrenaline, because control experiments showed that the sensitivity of the spleen strip to exogenous noradrenaline was not impaired by pronethalol. Pronethalol potentiated rather than depressed the responses to noradrenaline. Pronethalol is also known to inhibit noradrenaline uptake, and this again was suggested as a possible explanation for the potentiation.

9. Ouabain caused loss of potassium in the spleen strip, which indicated that downhill ion movement had taken place. Pronethalol delayed

both the downhill ion movement and the contraction caused by ouabain.

10. Increasing the calcium concentration of the bathing fluid from 2.5 to 5.0 mM reduced the height and prolonged the latent period of the ouabain-induced contraction. It was concluded that noradrenaline release was decreased. The sensitivity of the tissue was not impaired by the elevated calcium concentration; contractions caused by exogenous noradrenaline were enhanced.

11. Reducing the sodium concentration of the bathing fluid from 143 to 25 mM markedly reduced the amplitude and increased the latent period of the ouabain-induced contraction. No conclusions could be drawn from the reduced height of contraction, because contractions elicited by exogenous noradrenaline were also reduced. However, the increased latent period suggested that a longer time was required for ouabain to release noradrenaline.

12. Neither ouabain nor tyramine caused contraction of spleen strips which were depolarized by a high-potassium bathing fluid, but noradrenaline was as effective in the depolarized strips as it was in normal strips.

13. Depolarization by the high-potassium fluid caused a contraction consisting of two components in control strips. In strips from cats treated with reserpine the contraction consisted of one component and was significantly reduced in height. This suggested that noradrenaline release contributed to the contraction in strips from untreated cats.

14. All our observations were consistent with the idea that the ouabain-induced release of noradrenaline was due to inhibition of active sodium-potassium transport and its consequent downhill ion move-

ment. The long latent period between the addition of ouabain to the bath and the release of noradrenaline fits with the work of others showing that inhibition of sodium-potassium transport by cardiac glycosides is slow to develop. The inverse relationship between the latent period and the concentration of ouabain is similar to the work of others showing that the time required for cardiac glycosides to inhibit sodium-potassium transport is inversely related to concentration. Pronethalol opposed both the downhill ion movement and the release of noradrenaline caused by ouabain. An increased extracellular calcium concentration is known to stabilize the cell membrane in excitable cells and thus oppose the downhill movement of sodium into the cell. Increasing the calcium concentration of the bathing fluid antagonized the ouabain-induced release of noradrenaline. When less sodium was available to run downhill, i.e. in the low sodium medium, the time required for ouabain to release noradrenaline was markedly increased. A procedure known to decrease the inhibiting effect of cardiac glycosides on sodium-potassium transport, i.e. increased extracellular potassium, prevented the release of noradrenaline by ouabain. The effect of potassium was not irreversible since the releasing action of ouabain could be restored by returning the strips to Krebs-Henseleit solution.

15. In dogs, depletion of catecholamines by treatment with reserpine did not impair the ability of pronethalol to antagonize ouabain-induced arrhythmias. An intravenous injection of pronethalol (5 mg/kg) temporarily converted the arrhythmia to sinus rhythm. In some dogs sinus rhythm persisted after the third injection of pronethalol, while in other dogs the arrhythmia consistently reappeared after a few minutes. Infusions of pronethalol at a rate of 200 μg/kg/min abolished the arrhythmia, and sinus rhythm was sustained. Infusion at this rate into dogs treated with reserpine, but without ouabain-induced arrhythmias, increased cardiac automaticity. It increased the normal sinus rate and the frequency of firing of subatrial pacemakers which were elicited by vagal stimulation. It is concluded that the antagonism of digitalis-induced arrhythmias by pronethalol is due neither to prevention of catecholamine release nor to blockade of catecholamines at their receptor sites in the heart. Moreover, our results do not support the idea that the antiarrhythmic effect is due to an unspecific depression of cardiac automaticity similar to that caused by quinidine. It is likely that pronethalol antagonizes arrhythmias caused by digitalis by resisting downhill ion movement.

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