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CHAPTER I

INTRODUCTION AND STATEMENT OF THE PROBLEM

The cardiac glycosides occupy a peculiar position among the more important drugs applied to the practice of modern medicine in that they may be employed reliably and with confidence in the treatment of certain types of heart diseases for which they possess a truly remarkable specificity. The fundamental nature of their therapeutic effect, which is a positive inotropic action, still remains obscure. Over one hundred years of inspired research effort has produced a great body of literature contributed to by physicians, physiologists, pharmacologists, biochemists, histochemists and organic chemists. It is true that much of this work has resulted in negative findings or yielded conflicting and confusing evidence regarding the cardiotonic activity of these drugs, but in many cases inconsistencies were later reconciled by rationalization of technical differences in methods. With the development of advanced procedures and with the progress in scientific equipment made in recent years, the pattern of results of studies on these drugs is now assuming a more uniform description. However, the site of action of the cardiac glycosides at the molecular level is yet to be

described. As the discussion of the work of others may reveal, the cardiac glycosides in addition to their cardiotonic properties appear to have far-reaching physiological effects, but these are lesser in degree compared to the heart activity. Nevertheless, the possibility may exist that these secondary effects, usually classified as "toxic" side-effects or sequelae of the primary cardiac action, could provide important information regarding the fundamental mechanism through which these drugs influence metabolism at the cellular level. This concept was the basis for the problem of this thesis.

Briefly, the problem was designed first to confirm the results obtained by others regarding the influence of cardiac glycosides on heart tissue slice metabolism, employing the Warburg manometric technique. Secondly, this method was applied to the effects of ouabain, a cardiac glycoside obtainable in pure, crystalline form (Merck), on a tissue other than heart, namely, the kidney cortex.

The purpose of the problem was to test the possible influence of ouabain on:

1. Heart slice metabolism
2. Anaerobic glycolysis of kidney cortex slices
3. Aerobic respiration of kidney cortex slices
4. The oxidation of various intermediates of the tricarboxylic acid cycle by kidney cortex slices
5. The barbiturate depression of respiration of kidney cortex slices
6. Calcium and potassium ion concentrations in the slice suspending medium

7. Respiration of kidney cortex slices after toxic *in vivo* doses of the drug

8. Respiration of kidney cortex homogenates.

CHAPTER II

A REVIEW OF THE PHYSIOLOGICAL PROPERTIES OF CARDIAC GLYCOSIDES

The therapeutic usefulness of cardiac glycosides in heart diseases was not recognized as such until the time of William Withering, an English physician who was interested in botany as a hobby. For an undetermined period before this, a potion containing over twenty different herbs had been used for the treatment of dropsy by a family in Shropshire, England, the recipe for which had been handed down from one generation to the next (Salter, 1952). Withering became interested in this potion because of its beneficial effects in congestive heart failure even though it produced nausea and vomiting. Testing each herb, he selected the leaves of the red foxglove, Digitalis purpurea L., as the active ingredient. In 1785, he published a monograph entitled "An account of the Foxglove and some of its Medical Uses; with practical remarks on dropsy and other diseases" (Withering, 1937). The diuretic effects of the extract from the leaves of the foxglove were overlooked. In 1799, John Ferriar pointed out that the primary effect of the Digitalis extracts was on the heart while diuresis was a secondary effect (Salter, 1952). For many years the dried, ground leaves of Digitalis purpurea were used in the treatment of various heart conditions without

any knowledge of the nature of the active substance or substances. Indeed, powdered digitalis, or a tincture thereof, is still used in standard medical practice as a treatment for congestive heart failure, auricular fibrillation and flutter, and paroxysmal tachycardia. The first compound to be isolated and described as a crystalline substance was digitoxin. This was accomplished by Nativelle and published in 1869 (Stoll, 1937). He designated these crystals as "Digitaline crystallisee". Cleota published the first critical pharmacological study of digitoxin in 1920. The work of the chemist, Arthur Stoll, showed that the active principles in the Digitalis extracts were sterol glycosides (Stoll, 1933), and the history of the isolation of the various glycosides is summarized in his book which discusses the chemical properties of these substances (Stoll, 1937). It soon became apparent that digitoxin was not the only substance in the leaves of Digitalis purpurea which had cardiotonic activity, and the isolation of others (gitoxin and gitalin) soon followed (Cleota, 1926; Windaus, 1928). Thus the simplest substances from the foxglove and other plants containing cardiotonic drugs which produced a positive inotropic response were found to be sterol glycosides (Stoll, 1951). More specifically, they are hydroxylactones of sterol hydrocarbons in which one hydroxyl group is connected with a sugar molecule or a chain of several sugars (Jacobs, 1933). They are structurally similar to the saponins, which are widely distributed in plants and which have an important surface tension effect in plant saps, a property not possessed by cardiac glycosides, and correspondingly, the occurrence of the latter is

restricted to a fairly small group of families of plants. Moreover, the distribution of cardiac glycosides in the plant itself varies from one type to another. In some cases they are in the leaves or seeds; in others, the bulb; and still others, the wood or bark. Ouabain (also called Strophanthin-G), the glycoside used in the experimental work of this thesis is found in the seeds of Strophanthus gratus and in the roots and bark of the tree Acocanthera ouabaio (Merck Index) and was widely used by primitive peoples as an arrow poison. The physiological activity of cardiac glycosides in the metabolism of plants apparently has been investigated very little.

The specificity of this group of compounds for the heart becomes unmistakably clear in reviewing the work of numerous investigators. The gross effects of cardiac glycosides on the heart have been well worked out, but the biochemical nature of this activity has not been solved.

Physiologically, the primary effects of these drugs are on the heart and the central nervous system. Their activity on the heart is by far the more important, therapeutically. They also have stimulatory action on smooth and skeletal muscles. These extracardiac properties of the glycosides are considered clinically as toxic effects because they are not ordinarily observed in the therapeutic range of doses. However, as pointed out previously, the possibility may exist that the influence of these drugs on other tissues may offer clues to their biochemical adjunct with cellular metabolism in the heart. The first section of this review deals with these non-cardiac effects; the second section is concerned with cardiac effects; and the third section reviews the

small amount of work done with glycosides on kidney metabolism.

Extra-cardiac Effects Of The Cardiac Glycosides

The effects on the brain at lower dosages are to increase vagal tone (Lendle, Mercker and Rorh, 1953) due to the response of the medullary centers. This may result in nausea. Vomiting may be due to gastric irritation if the route of administration is oral (Gold, Kwit, Cattell and Travell, 1942; Borison, 1952). The true toxic effect causing nausea is not gastritis as Dresbach (1939, 1941) has shown that a nausea response is observed in the eviscerated cat after parenteral administration. The respiratory center may also be stimulated. As the dosage increases beyond this, visual effects have been reported including xanthopsia, scotomata and amblyopia, but these symptoms are reversible (Langdon and Mulberger, 1945). Severe poisoning may cause general paralysis. Smooth muscles have been observed to contract slightly in the therapeutic range of doses. This may have a beneficial effect by increasing the blood pressure along with the positive inotropic response in patients with failing hearts (Bucherl and Schwab, 1952). The effects on skeletal muscle resemble those which will be described for the heart, but are not nearly as pronounced. These effects are similar to the events that occur when there is a loss of potassium from the muscle cells (Gutman and Cattell, 1940; Calhoun and Harrison, 1931; Cattell, 1938; Wedd, 1939), or a loss of glycogen (Bamskov, v. Kaulla and Maurath, 1941). The blood volume may be reduced in the normal man and is the usual case when there is congestive failure of the heart. The ingestion of 2.2 mg of digitoxin by normal humans caused a reduction of plasma

volume from an average initial value of 48.1 ml/kg to a minimum average value of 44.0 ml/kg. Similarly, the hematocrit was increased from 38.2 to 40.1 percent, and blood volume was decreased from 77.9 ml/kg to 73.5 ml/kg (Schwab and Weber, 1953). Strophanthin-k and digitoxin and their aglycones, (sterol moieties) strophanthidin and digitoxigenin, have been shown to inhibit the active transport of potassium and sodium through the erythrocyte in concentrations of 0.1 to 10 gamma/ml, although these same concentrations have no significant effect on the anaerobic glycolysis or oxygen consumption of red cells (Schatzmann, 1953). Administration of 0.25 mg of strophanthin causes an initial increase in blood sugar followed by a fall to below average values, but the variations are within the range of normality (Wolff, 1954).

Because of the structural similarity of cardiac glycosides to the steroid hormones, a number of studies have been made regarding their hormone-like properties. It has often been observed that in addition to their own specific effects, some hormones possess properties resembling those of other hormones. Guinsburg demonstrated in 1928 the adrenal cortical hormone-like effect of strophanthin upon plasma potassium and sugar levels. This was confirmed by Zwemer and his collaborators in 1940, who also showed that certain cardiac glycosides could protect against insulin shock. Bamskov and co-workers demonstrated in 1941 that digitalis preparations caused a marked decrease in the glycogen content of the liver, skeletal muscles and the heart of rats. They also concluded that digitalis substances affect the basal metabolism. Small doses lower the metabolic rate as if it had already been raised above normal by thyrotropic hormone, but large doses, on the

other hand, act like thyrotropic hormone. Digitalis also had an inhibitory effect on the loss of body weight produced by thyrotropic hormone. After prolonged oral administration of small doses, Kuusisto reported in 1952 that cardiac glycosides have an inactivating effect on the thyroid of the guinea pig. The blood cholesterol level was lowered, but there was no effect on the animals' oxygen consumption. The thyroid glands of the treated animals showed a reduction in the height of the secretory epithelial cells, and an increased amount of colloid, with a decrease in the functional activity of the epithelial cells. Because the metamorphosis of the frog tadpole is a sensitive reflection of thyroid activity in these animals, another group tested these drugs on the development of frog larvae (Halonen, Kuusisto and Koskelo, 1952). Strophanthin, in a concentration of 1:400,000 retarded tadpole metamorphosis, but did not counteract the acceleration of development induced by treatment with thyroxin. The glycoside did produce development of tadpoles of larger than average size, and these results point to a lowering of thyroid gland function, perhaps by the inhibition of thyrotropic hormone.

There is a possibility that a relationship exists between the adrenal cortical steroids and the cardiac glycosides regarding their physiological effects. From clinical and experimental studies, a definite degree of antagonism appears to exist between the thyroid and adrenal cortical hormones. The hyperthyroid patient exhibits some of the symptoms which are criteria for the administration of digitalis drugs (tachycardia and various degrees of heart failure). Because of this, studies were made on experimentally produced hyperthyroidism in

animals which were given cardiac glycosides to test for an adrenal-cortical-like antagonism of the toxic condition (Kinsell, Zillesson, Smith, and Palmer, 1942). They found that the glycosides exert a protective effect against at least some part of the stimulation of metabolism due to administered thyroxin. It was shown that an increase in the metabolic rate decreased the tolerance for digitalis (Plummer et al, 1925) and it was pointed out that there may be danger of cerebral edema when digitalis is administered to hyperthyroid patients, possibly due to a loss of potassium with replacement by sodium in the brain cells. Usually, in the absence of hyperthyroidism, therapeutic doses of digitalis tend to restore the normal balance between the intracellular and extracellular potassium, while toxic doses have the opposite effect. The effects of cardiac glycosides on potassium balance may explain some of the similarity to adrenal cortical steroids which also affect potassium in a like manner (Ferrebee, 1943). On the other hand, it was shown that the glycosides do not induce the adrenal cortical effect of eosinopenia produced by compound F (Chen, Anderson and Rose, 1952).

Gynecomastia attributable to digitalis drugs is another hormone-like effect. Male patients receiving various forms of digitalis for congestive heart failure have been reported to develop breast swelling which subsided after discontinuing treatment (LeWinn, 1953). This may be a manifestation of a similarity to estrogenic steroids.

Another relationship of these drugs is seen with histamine. Administration of digitoxin depresses the blood level of histamine. An increase in the blood level of histamine will greatly potentiate the lethal effect of digitoxin (Sjoerdsma and Kun, 1948).

Cardiac Effects Of The Cardiac Glycosides

The greatest emphasis in fundamental research on the actions of cardiac glycosides has been on cardiac tissue in all types of preparations, and has been in progress over a century and a half, resulting in a large body of literature. Information has been selected from this literature which characterizes the metabolic effects of this group of drugs on cellular functions of the heart muscle since the problem of this thesis is primarily at the tissue level. In order to define more clearly the properties of these drugs on cardiac tissue, pertinent work at the systemic and organic level has been included. The work on the heart falls into three general categories; 1) studies on the whole organ, either *in vivo*, or in heart-lung preparations or similar methods, 2) studies on extracted constituents of cardiac muscle, such as actomyosin; and 3) studies on the influence of these drugs on the energy metabolism of the heart. These subjects are discussed in the same order.

It has been demonstrated that digitalis has quite different effects on the normal heart as compared with those on the failing heart. Digitalis decreases the size of the normal heart to a subnormal value, thereby decreasing the cardiac output. Similarly, the effect on the failing heart is also to decrease its size, but in this case the enlarged heart is brought to a near-normal condition, with an increase in systolic force which empties the organ more efficiently and increases the cardiac output (Schnitkner and Levine, 1937). The drugs do not act by slowing the heart primarily, for as Wiggers pointed out in 1931, this

would eventually kill the patient since the failing heart is working at a great rate in order to compensate for low cardiac output due to weakened contractile force. In practice, however, it is recognized that slowing often does occur with therapeutic doses of cardiac glycosides. This is attributed to the vagal effect of these drugs and to an increased excitability of the carotid sinus (Ferris, Capps and Weiss, 1935). Digitalized persons exhibit an increased P-R interval, which is the electrocardiographic manifestation of delayed auriculoventricular conduction (Donhardt, 1951; Levine, Nahum Seller and Sikand, 1951). This is due partly to the vagal effect and partly to the effect of the drug on the bundle of His (Salter, 1952). It appears that the beneficial effect of digitalis compounds is through the increased force of contraction by direct action on the cardiac muscle. This eventually relieves pulmonary and venous congestion, decreases the tone of the Bainbridge reflex and thereby allows the heart to slow to a more normal rate of beating.

Perfusion studies have been made on hearts removed from various animals. Isolated hearts of rats, guinea pigs, rabbits, dogs and cats were perfused with Ringer-Locke solution containing digitoxin labeled with radioactive carbon. The study showed that the glycoside is rapidly fixed in the myocardium early in the perfusion, the rate depending upon the species of animal. Sixty percent of the total radioactivity could be extracted from the heart as unchanged digitoxin (Sjoerdsma and Fischer, 1951).

The effect of glycosides on the refractory period of the heart has been investigated. It was found that in the anesthetized dog,

cardiac glycosides shortened the refractory period of the auricle due to the action of the vagus, and also shortened that of the ventricle, but not obviously until approximately 40 percent of the lethal dose had been injected (that is, the quantity of digitalis fixed by the heart then approached the level at which the first irregular systoles appear). In the same study, it was determined that the refractory period of the auriculo-ventricular propagation tissue was progressively increased to the point of complete A-V block (Mendez and Mendez, 1953).

Ouabain has some effect on coronary circulation. In doses of 10 gamma/kg, there was a decrease in the coronary sinus outflow following the second or third dose in dogs. These averaged 25% below the control levels. At the fifth or sixth dose, there is a marked increase in flow averaging 96% above controls. The changes in coronary sinus outflow showed no correlation with changes in blood pressure or heart rate. Ventricular fibrillation usually occurred following the tenth or eleventh dose (Sherrod, 1951; Page, 1951).

In experiments where fatigue of the heart was produced by constricting the main pulmonary artery, it was found that, in dogs, ouabain permitted more constriction of the artery and at the same time postponed the physiological fatigue from over-loading, and thus appears to have a favorable effect on the dog's heart under stress (Selzer, Lee, Goggans and Gerbade, 1953). Ischemia appears to make the heart more sensitive to the effects of digitalis (Del Pozo, 1950). The glycosides and their aglycones, however, have been shown to vary in regard to the type and intensity of these common properties (Iwamoto, Bell, Laquer, Carr, and Krantz, 1947).

In the failing heart condition where blood pressures are below normal, digitalis has been demonstrated to cause an average increase of 43 mm Hg pressure (Cotton and Walton, 1951).

The effect of ouabain on the guinea pig heart at different ages has been studied. The intravenous lethal dose of cardiac glycosides decreases progressively with the weight of the animal, the total decrease amounting to 15 to 20 percent (Goldberg, 1949). This work was later confirmed by another investigator who also found that it required twice as much ouabain to stop a 20-25 day-old isolated guinea pig heart as for the adult heart, but there was no difference in effect on hearts with regard to sexes of the same age (Wollenberger, Jehl and Karsh, 1953). By comparison, it has been shown that it required 50 percent more ouabain to cause cardiac arrest in the isolated heart of the female rat than for the male (Holck and Kumura, 1951). A seasonal variation in the response to ouabain exists in some animals (Chen, Henderson and Robbins, 1953), but data regarding this effect in guinea pigs was not included.

The presence of certain ions has been known for some time to alter the effect of cardiac glycosides on the heart. For example, if the concentration of calcium in a perfusion medium is below the physiological amount, the contractile response is small, but can be restored to normal by addition of ouabain. Calcium has a synergistic effect on the inotropic action of cardiac glycosides (Salter, Sciarini and Gemmel, 1949). Another group obtained evidence to support the hypothesis that the metabolic reactions involved in oxygen utilization of the heart are not essential for the inotropic action of calcium or

cardiac glycosides, but rather that the critical reactions are those concerned with the utilization of high energy compounds such as creatine phosphate and adenosine triphosphate (ATP) (Ellis and Anderson, 1951). Potassium appears to have an effect opposite that of calcium. An excess of extracellular potassium decreases the cardiac activity of glycosides, while a deficiency of potassium enhances their effects (Wilbrandt and Caviezel, 1954). Supporting this finding is the fact that in humans being treated for congestive heart failure, the toxic effects of administered digitalis could be terminated by oral or intravenous potassium salts, and similarly in digitalized patients with uremia, the extraction of potassium by hemodialysis was associated with the development of auricular tachycardia and block (Lown, Wyatt, Crocker, Goodale and Levine, 1953). The onset of toxic arrhythmias in the isolated cat heart due to ouabain was delayed or prevented by addition of potassium ions (Garb and Venturi, 1954). In addition, there is some evidence for the theory that activities of cardiac glycosides are concerned with their ability to chelate intracellular copper (Chenoweth, Bennett and Parry, 1951) required for the activity of some enzyme systems. The relation of the action of cardiac glycosides to ion concentrations may support the suggestion that these drugs act on the cardiac cell membrane (Wollenberger, Jehl and Karsh, 1953).

Studies of the effects of cardiac glycosides on heart muscle in tissue culture have been performed. In one case, ouabain had no stimulating effect on the growth or "movement" of heart cells in tissue culture even though there is a greater susceptibility of such cultures to toxic effects of the drug than in the whole organism (Orzechowski,

1935). This supported by later work in which concentrations of ouabain from 10^{-5} M to 10^{-8} M did not stimulate tissue cultures from the chick (Heubner and Schreiber, 1939-1940). On the other hand, inhibition of the pulsation of heart fragments in culture as well as their proliferation in the presence of ouabain has been reported (Varga, 1931).

The second of the three categories of literature on the heart surveyed is that concerning the protoplasmic constituents of myocardial tissue. The influence of ouabain has been determined on actomyosin extracted from beef heart (Edman, 1950, 1951a, 1951b; Helmreich and Simon, 1952). They found that concentration of 10^{-5} M and 10^{-6} M ouabain stimulated the activity of myosin-ATPase. Another group has shown that ouabain causes a more rapid and more complete contraction of prepared actomyosin fibers when such fibers are in a medium containing ATP (Robb and Mallov, 1953). Further, they demonstrated that this action was more pronounced on heart muscle actomyosin than that extracted from skeletal muscle. Other glycosides tested apparently had no effect on the ATPase activity of various cell fractions (Langemann, 1953).

The third category is concerned with the effects of cardiac glycosides on the energy metabolism of the heart. The striking effect of such small doses of these drugs on the physiological processes of animals led many investigators to seek the primary site of action at the level of metabolic reactions within the cells. The intracellular distribution of digitoxin in cardiac tissues has been analyzed. The results indicated that the glycoside was concentrated in the soluble portion of cell constituents, with more than 85 percent recovered from that fraction, while only 3 percent was recovered from the mitochondria

(St. George, Friedman, and Ishida, 1953). The possible influence of cardiac glycosides on the carbohydrate metabolism of the heart has been investigated. In utilizing carbohydrate anaerobically, the end product of glycolysis in mammalian hearts has been shown to be the production of stoichiometric amounts of lactic acid only, but it has been pointed out that the heart is essentially an aerobic organ, and its respiration is profoundly disturbed by anoxic conditions which would have little or no effect on other tissues excepting brain perhaps (Barron, Sights and Wilder, 1953). Aerobic energy metabolism as affected by cardiac glycosides has also been investigated. The theoretical and experimental basis for studying the energetics of the failing heart was established by studies of Starling and his collaborators who demonstrated that the mechanical energy set free in the contraction of the heart depends on the diastolic volume, which in turn depends upon the initial length of the heart muscle fibers and also determines the amount of oxygen consumed (Patterson, Piper and Starling, 1914). The validity of this postulate was later shown to be subject to doubt in studies where this relationship did not hold true (Bing, Hammond, Handelsman, Powers, Spencer, Eckenhoff, Goodale, Hafkenschiel and Katz, 1949).

The interruption of the energy metabolism of the heart may occur in one or both of two phases of the process. The heart may be assumed to be a machine for the conversion of chemical energy into mechanical energy. The first stage of this conversion is the liberation of energy from carbohydrates through glycolysis and respiration. The second stage is the utilization of the energy produced, which, in the heart, brings about contraction. In the failing heart, energy is

liberated normally, but not utilized properly, resulting in a loss of mechanical efficiency (Wollenberger, 1949). In some manner, cardiac glycosides restore the ability to use this energy efficiently. In the same dosage, they have little effect on the normally functioning heart except to increase the oxygen consumption considerably (Eismayer and Quincke, 1930). What becomes of the extra energy produced by this increase in oxidation is not clear. It is also not clear whether the mechanism by which these drugs produce their positive inotropic effect is basically the same as that causing the increase in respiration; therefore, it cannot be definitely decided whether these drugs act at the energy production level or the energy utilization level, but the latter seems more likely in view of the investigations reported.

There can be little doubt that the glycosides profoundly increase the respiration of tissue slices of heart; yet, at the same time, they have little or no effect on heart homogenates (Levy and Libert, 1946, 1947; Libert, 1946, 1946a; Wollenberger, 1947, 1947a, 1949; Finklestein and Bodansky, 1948). The increases in respiration range up to 200 percent of the control slice respiration, and the increased rate is sustained for as long as four hours. The concentrations of drugs used were in the therapeutic or at least non-toxic range. A similar, but less pronounced increase of respiration has been found for brain cortex slices, but, again, not for brain homogenates (Wollenberger, 1947a, 1950. One group failed to find any effect at all on brain tissue (Langemann, Brody and Bain, 1953). The fact that in the failing heart there is a normal amount of ATP and creatine phosphate, lends support to the idea that the defect in this condition is concerned with

the utilization of these available energy supplies.

The influence of cardiac glycosides on some of the oxidative enzymes of carbohydrate metabolism has been investigated. Anaerobic glycolysis of brain is strongly inhibited (Wollenberger, 1950) and has been reported so for rat heart tissue (Libert, 1946a), but not for guinea pig heart, which glycolyzes very little anyway in slice preparations. This inhibition of glycolysis can be largely prevented but cannot be reversed by 0.04 M nicotinamide, which may suggest that these drugs stimulate a diphosphopyridine nucleotidase (DPNase) activity in the cell (Wollenberger, 1950). Blockade of glycolysis with sodium iodoacetate, which inhibits the oxidation of 3-phosphoglyceraldehyde does not interfere with the positive inotropic property of the glycosides (Ellis and Anderson, 1953). Another group found that glycosides did not affect glycolysis of heart muscle slices nor did they have any influence on the oxidation of phosphoglyceraldehyde or lactic acid with purified enzyme systems (Reiter and Barron, 1952).

Enzymes of the tricarboxylic acid cycle have also been studied with regard to their sensitivity to the cardiac glycosides. It has been reported that a concentration of 1 X 10^{-4} M ouabain produced a maximum stimulation of 20 percent within one-half hour in heart slices utilizing oxalacetate as substrate, but was followed by a 37 percent inhibition at the end of three hours. No effect was noticed when the substrate was succinate, malate or acetate (Herrmann, 1950). One group reports that even such large concentrations as 0.004 M ouabain have no influence on the utilization of succinate, malate, lactate, or on endogenous respiration of rat heart homogenates (Saunders, Webb, Leyden and Thienes,

1950). On the other hand, digitoxin has been alleged to show definite inhibitory effects on the heart muscle oxidases of rabbit, cat, dog and calf heart muscle (Macht, 1951). These findings are of interest in view of the fact that it has been demonstrated that the tricarboxylic acid cycle actually does operate in human cardiac muscle (Burdette, 1952).

Because of the importance of oxidative phosphorylation in energy metabolism, investigations of the effects of cardiac glycosides on phosphate metabolism have been done. Although toxic doses of glycosides deplete stores of creatine phosphate in heart muscle (but without affecting the ATP content), concentrations at therapeutic levels are without influence on creatine phosphate, ATP, or inorganic phosphate. Dinitrophenol increases oxygen consumption by uncoupling phosphorylations from oxidations. Cardiac glycosides have been shown not to stimulate respiration by such an uncoupling. Oxidative phosphorylation is apparently refractory to these drugs at any concentration (Herrmann, 1950; Wollenberger and Karsh, 1952; Reiter and Barron, 1952; Wollenberger 1950, 1951). While relative amounts of high-energy compounds appear to be unchanged by therapeutic concentrations of glycosides, it has been reported that there is an increase in the rate of radioactive phosphorus turnover in rat hearts treated with sub-toxic doses of digitoxin (Alstrom, 1954).

Because the cardiac glycosides exert their most striking effect on tissues with depressed oxygen consumption, barbiturates have been used to bring about artificial depressions of cardiac muscle. Barbiturates are known to inhibit aerobic oxidation perhaps by competing with pyruvate for dehydrogenase surface (Persky, Goldstein and Levine, 1950)

and are also known to interfere with the generation of high-energy phosphate in brain (Quastel, 1952). Concentrations of pentobarbital causing 50 to 80 percent reduction in the amplitude of auricular contractions in the isolated guinea pig heart decrease the rate of potassium loss from these preparations. The decrease in potassium ion permeability produced by the barbiturate can be partially or completely prevented by relatively small additions of acetylcholine and ATP to the suspending medium (Holland, 1954). It had already been pointed out earlier that the most likely mechanism of barbiturate interference (as well as calcium deficiency) on the heart is the impairment of the processes intervening between the passage of the action potential and the onset of the contractile response (Krogh, Lindberg and Schmidt-Nielsen, 1944, Wilbrandt and Koller, 1948). These facts are relevant to the finding that non-toxic concentrations of ouabain are able to reverse rapidly the depression of cat heart muscle respiration by pentobarbital (Langemann, Brodie and Bain, 1953), and also has a bearing on the report mentioned earlier that a deficiency of extracellular potassium will sensitize the heart to the inotropic effect of the glycosides (Wilbrandt and Caviezel, 1954).

Cardiac Glycosides And The Kidney

While the diuretic effect of cardiac glycosides is usually attributed to improved hemodynamics with the result of increasing blood supply to the kidneys, recent studies on the effects of glycosides on the renal excretion of plasma electrolytes and on the metabolism of kidney cortex indicate that this may not be the true picture. After

administering 0.35 mg of strophanthin-k to normal subjects, their renal sodium, potassium and chloride excretion rose significantly while renal water excretion remained unchanged. The plasma concentration of sodium decreased, while the plasma concentration of potassium increased, with plasma chloride remaining unchanged (Dorrie, Goltner and Schwab, 1954). It is now known that sodium and potassium ions exchange rapidly between cells and body fluids, and that the observed concentration gradients are not due to impermeability of cell membranes (Krogh, 1946; Ussing, 1949). The production and maintenance of physiological concentration gradients of sodium and potassium in isolated kidney cortex slices requires respiratory energy (Whittam and Davies, 1954). Strophanthin-k injected into mice caused a diuresis which was found to be of the filtration type (Frey, 1952). Cardiac glycosides have been shown to be fixed in the kidney as well as in the heart and liver (Giertz, Hahn, and Schunk, 1954). The diuretic effect of digitoxin develops slowly to a maximum over a period of 48 to 72 hours (Greiner, Gold, Rao, Washaw, Hiroshi and Otto, 1955). The incubation of rat kidney slices in a physiological medium containing 0.001 gamma/2 ml of strophanthin-k causes an increase in Q_{O_2} of 15 percent, while concentrations ranging from 1 to 100 gamma/2 ml decreases the Q_{O_2} by 30 percent. There was apparently no effect on the respiration of kidney homogenates, indicating that the intact tubule cell is required for the activity (Frey, 1952, 1953). With the use of radioactive phosphorus, it was shown that subtoxic doses of digitoxin decreases the rate of phosphate metabolism in the rat kidney (Alstrom, 1954).

The finding that physiological amounts of cardiac glycosides

depress the energy metabolism of kidney tissue may be relevant to the concurring changes in electrolyte excretion and so-called diuresis.

CHAPTER III

MATERIALS AND METHODS

Normal adult guinea pigs were used in all experiments. The animals were maintained with a standard rabbit pellet food (Superior) fed ad libitum, supplemented at regular intervals with Vitamin C or fresh vegetables. Their weight at time of sacrifice was in the range of 450 to 500 grams. After stunning the animals with a sharp blow on the head, they were exsanguinated by severing the blood vessels of the neck. Depending on the experiment to be performed, the heart or kidneys were quickly removed to a beaker of iced Krebs-Ringer-Phosphate or Krebs-Ringer-Bicarbonate solution (Umbreit et al, 1949). After chilling thoroughly, the tissue was sliced according to the standard free-hand method usually used for such preparations described by Deutsch in 1936. The tissue was placed on a frosted glass plate which was lying on ice. A frosted glass slide, chilled by immersion in the ice cold buffer, was placed upon the organ and gentle pressure was applied. The cutting instrument was a new, carefully washed double-edged razor blade firmly attached to a wooden handle. The blade was passed through the tissue just beneath the frosted glass slide. When the slide was lifted, the tissue slice remained with it and could be gently removed with flat-tipped forceps to fresh, cold physiological medium (Krebs-Ringer) which had been previously aerated with oxygen. The thickness of the slice was

important because the rate of diffusion of oxygen into the tissues must be sufficient to meet the requirements of all the cells. Thus, a slice respiring in air may be no thicker than 0.2 mm, while one respiring in an atmosphere of 100 percent oxygen may be as much as 0.5 mm in thickness (Warburg, Kubowitz and Christian, 1930). The thickness of any slice could be accurately estimated by placing it in a petri dish which had squared millimeter paper beneath it. By spreading the slice flat on the bottom of the dish, the area could be determined by counting the number of squares it covered. This figure, divided into the weight of the slice (which is a close measure of the volume of tissues) gave the thickness (Umbreit Burris and Stauffer, 1949). In practice, investigators have found that after some experience the thickness of a slice could be controlled in free-hand slicing by the appearance of the blade passing through the tissues, and by the way the slices curled up on lifting them from the frosted glass slide as well as by the manner in which they completely flattened out when placed in a physiological medium. Slices cut in this fashion were found by spot-checking to vary between 0.3 and 0.5 mm in thickness. This was a desirable range for manometric studies employing 100 percent oxygen atmosphere. All instruments and solutions were kept below 5^{o} C. insofar as possible by placing them on ice, with frequent immersion of the glass slide and cutting blade into ice-cold Krebs-Ringer-Phosphate buffer. The first slice from each organ was discarded. In slicing the kidneys, care was taken only to include the cortical portion in the section.

After completion of the slicing, several tissue sections with a total weight averaging 175 mg were placed into each Warburg reaction

vessel which had been previously prepared with media and additives and placed on ice. In weighing the slices, filter paper was used to absorb excess fluid from the tissue before placing them in the pan of the torsion balance. The handling of slices during these manipulations was done very carefully in order to minimize damage to the tissue.

The suspending medium used is dependent on the nature of the experiment to be performed. In studies where the rate of anaerobic glycolysis was being determined, the choice of medium was Kreb-Ringer-Bicarbonate solution. When tissues oxidize glucose in the absence of oxygen, the end-product of metabolism is lactic acid. In a bicarbonate buffer, lactic acid is neutralized, releasing an equivalent of carbon dioxide. The gas phase used with the bicarbonate was 5 percent carbon dioxide and 95 percent nitrogen. The carbon dioxide of the gas phase was in equilibrium with that of the suspending medium. Any excess carbon dioxide in this medium produced by the neutralization of lactic acid formed by the tissue will result in the displacement of that amount of carbon dioxide back into the gas phase. In the Warburg respirometer, the volume and temperature are kept constant, so that any change in the amount of gas will result in a proportional change of pressure. Therefore, the amount of carbon dioxide produced by the neutralization of lactic acid can be measured by following the increase in pressure within the system as measured on the manometer to which the reaction vessel is attached.

To be certain that the substance being neutralized was actually lactic acid and not an accumulation of one of the intermediary compounds, total lactic acid was determined for each vessel of the anaerobic

experiments using the method of Barker and Summerson (1941). This colorimetric method has been shown to give an accurate evaluation for lactic acid in biological materials. The technique fundamentally involves the oxidation of lactic acid to acetaldehyde, and the reaction of acetaldehyde with para-phenylphenol, which produces a violet color according to the amount of acetaldehyde present. The color intensity is measured by the transmission of light at 565 mu in a spectrophotometer. If the conversion of glucose to lactic acid is not inhibited at some intermediate point, the number of moles of lactic acid determined is expected to agree with the number of moles of carbon dioxide produced during the experiment.

For measuring oxygen consumption, Krebs-Ringer-Phosphate solution was the choice of medium, employing an atmosphere of 100 percent oxygen. In this technique, the amount of oxygen used was measured by a corresponding drop in the gas pressure within the system. In order to avoid the masking of the utilization of oxygen by the evolution of carbon dioxide from the decarboxylation of various intermediates, alkali was placed in the center well of each vessel to absorb the CO_2 as it was produced. Admittedly, it was less physiological to use an atmosphere of pure oxygen than to use one containing some carbon dioxide, but this disadvantage was offset by the fact that tissue slices respiring in air need to be so much thinner that the proportion of damaged or destroyed cells to intact ones is very high.

In most of the experiments with tissue slices, ouabain was dissolved in a small amount of the suspending medium and pipetted into the side-bulb of the flask to be tipped into the main chamber at a

designated time. The experiments requiring sodium pentobarbital were done in the same manner, except that the barbiturate usually goes into the main chamber at the very first. Departures from this scheme and the reasons for doing so are clarified in the section describing results of these experiments.

All substrates were freshly prepared at the time of each experiment from reagent grade materials. Sodium alpha-keto-glutarate, sodium pyruvate, sodium fumarate and sodium malate were prepared from their respective acids by neutralization with sodium hydroxide.

All tissue slice experiments were incubated at a water-bath temperature of 38° C.

Homogenates of kidney cortex were used in some of the work and these were prepared by grinding the tissue in buffered, isotonic KCl (0.154 M KCl plus 8 ml of 0.04 M $KHCO_3$ per liter). Ten percent homogenates were used, to which cytochrome c, adenylic acid, inorganic phosphate and $MgCl_2$ were added (Umbreit, Burris and Stauffer, 1949). The grinding was accomplished with a mortar and pestle, and all manipulations were performed in the cold. Incubation of experiments using homogenates were all done at 31° C. in 100 percent oxygen.

Gas exchange in all cases was maintained for at least three minutes by attachment of all manometers simultaneously through a manifold to compressed gas tanks.

Manometer readings were taken every ten minutes for from two to four hours. The duration of incubation in most experiments was two hours. Oxygen consumption or carbon dioxide evolution was calculated on the basis of 100 milligrams of tissue (wet weight) per unit time.

After establishing the techniques so that experimental results became reproducible, each experiment was performed in duplicate or triplicate at least three times, and in most cases five times or more.

Certain minor departures from the procedures outlined occurred at several points in the experimental work, but these are accounted for in the discussion of the results.

CHAPTER IV

PRESENTATION OF RESULTS

Effect of Ouabain on the Anaerobic Glycolysis of Guinea Pig Kidney Cortex Slices

The results of a typical experiment are shown in Figure 1. The carbon dioxide liberated by anaerobic glycolysis of these slices is evolved from the bicarbonate buffer at the same rate after tipping in ouabain at 30 minutes as it is in the controls which do not receive the cardiac glycoside. The results were identical in all of the experiments, indicating that ouabain does not influence kidney cortex glycolysis. Aliquots of the suspending medium, removed at the beginning and at the end of the experiments, were analyzed for lactic acid. The difference between the two values represents the amount of lactic acid produced during the period of incubation. Since one mole of lactic acid will liberate one molar equivalent of carbon dioxide, the molar ratio of lactic acid to carbon dioxide should, theoretically, be 1. Table 1 shows the results of part of the analyses:

Table 1

Ouabain	Moles Lactic A.	Moles CO_2	Ratio
-	11.0	9.3	1.2
-	9.1	9.6	0.9
5×10^{-4}M	8.6	9.4	0.9
5×10^{-4}M	11.4	9.0	1.3
5×10^{-4}M	8.2	9.1	0.9

EFFECT OF OUABAIN ON THE ANAEROBIC GLYCOLYSIS OF GUINEA PIG KIDNEY CORTEX SLICES

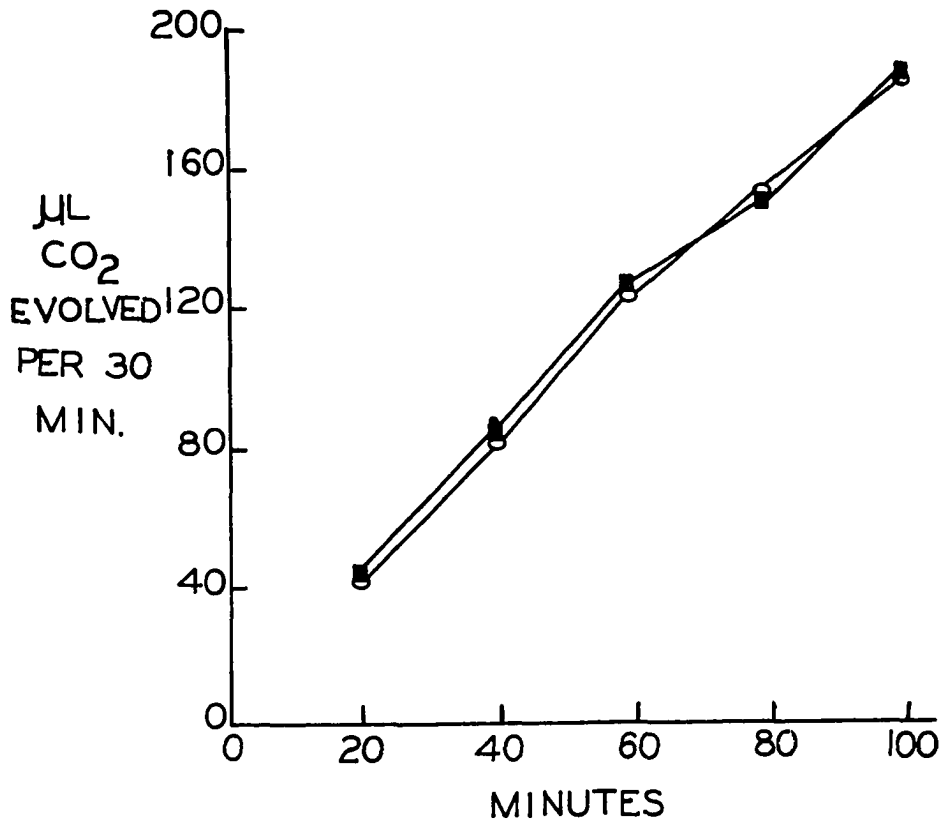

Figure 1. Carbon dioxide evolved per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.06 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.02 M Bicarbonate Buffer, pH 7.4, 21 parts. Solid squares: Ouabain, 5 X 10^{-4} M added at 0 minutes. Circles: None.

Considering that there is a margin of error in both the manometric technique and the lactic acid determination, the figures indicate that there is no difference in the ratio of lactic acid to carbon dioxide whether ouabain is present or not. This supports the conclusion that ouabain (5×10^{-4}M) does not interfere with anaerobic glycolysis of kidney cortex slices.

Effect of Ouabain on the Barbiturate Depression of Guinea Pig Heart Slices

The respiration of guinea pig heart slices under these conditions is low. Initially the rate of the control vessels is about 15 to 20 microliters/30 minutes/100 mg wet weight of tissue. It falls in about one hour to approximately half of this value. This diminishing of respiration cannot be due to depletion of substrate, for the concentration of glucose in the medium is 1.1×10^{-2} M, which is adequate for the length of time of the experiment. In spite of the small amount of respiration, it may be seen that sodium pentobarbital in a final concentration of 1.0×10^{-3} M produces a rapid drop in respiration. Curve 2 of Figure 2 represents the type of respiration obtained with barbiturate-depressed heart slices. Curve 3 shows the typical stimulation of respiration obtained by adding 5×10^{-4} M ouabain after 80 minutes of depression. The increase in respiration brings the rate above the existing control rate (Curve 1). These experiments were not continued long enough to determine how long this stimulation is maintained, but the data are consistent with the work of Wollenberger in that a reversal of the barbiturate depression is brought about by ouabain.

Effect of Ouabain on the Barbiturate-Depressed Respiration of Guinea Pig Kidney Cortex Slices

It was considered of interest to know whether the reversal of barbiturate-inhibited oxygen consumption brought about by 5×10^{-4} M ouabain which occurs in the heart muscle would also be true for kidney cortex slices. Since it has already been shown that reversal of such a depression does not occur in brain slices (Langemann et al, 1953), it may be that this property of the glycosides is another one of its specific effects on heart muscle. Figure 3 shows the results obtained from endogenously respiring slices. Here, 5×10^{-4} M ouabain, added to the depressed tissue after 62 minutes, augments the depression (Curve 4). It was noted in these experiments that ouabain alone produced a severe depression of respiration (Curve 3). Figure 4 shows a similar experiment with a substrate (1.1×10^{-2} M glucose). Here, too, ouabain augmented the depression of respiration already caused by pentobarbital. Curve 3 shows that the addition of ouabain to undepressed slices results in a marked depression of respiration. It became clear that the glycoside does not reverse barbiturate depression, but also acts as a depressant itself.

Effect of Ouabain on the Oxidation of Various Intermediates of the Tricarboxylic Acid Cycle

Because the previous study indicated that ouabain is interfering with aerobic respiration, perhaps by inhibition of a respiratory enzyme, the utilization of various tricarboxylic acid cycle intermediates was tested.

EFFECT OF OUABAIN ON THE BARBITURATE-DEPRESSED RESPIRATION OF GUINEA PIG HEART SLICES

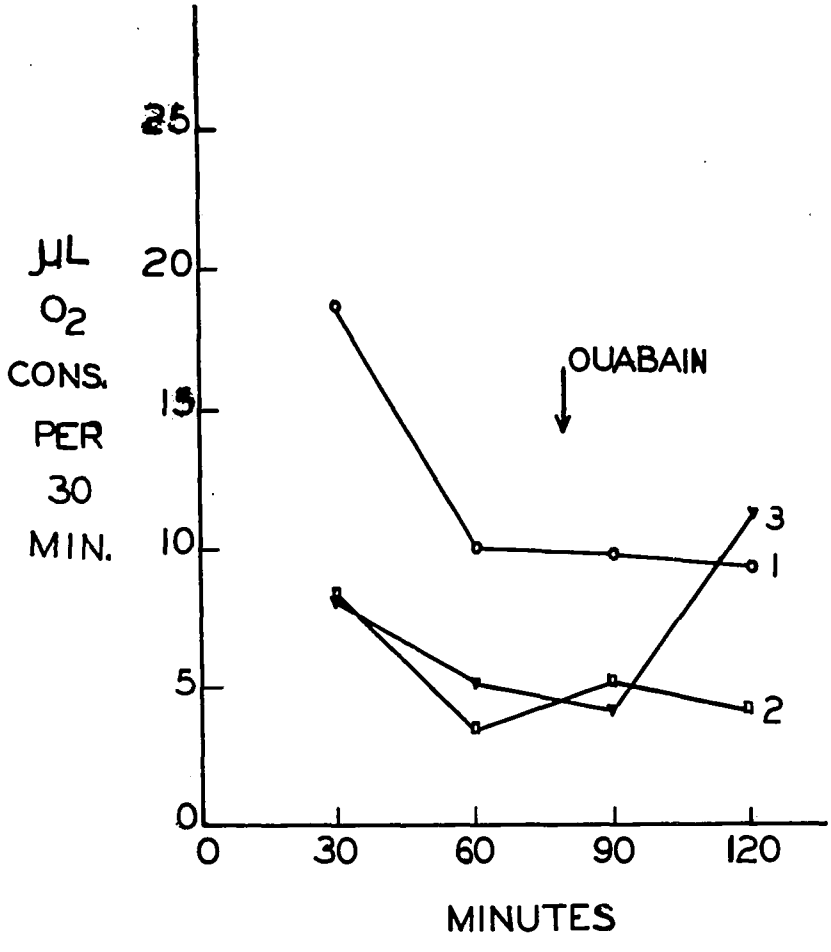

Figure 2. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.06 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. Final concentration of additives: 3: 1×10^{-3} M sodium pentobarbital at 0 minutes, 5×10^{-4} M ouabain at 80 minutes. 2: 1×10^{-3} M sodium pentobarbital at 0 minutes. 1: None.

EFFECT OF OUABAIN ON THE BARBITURATE-DEPRESSED RESPIRATION OF GUINEA PIG KIDNEY CORTEX SLICES

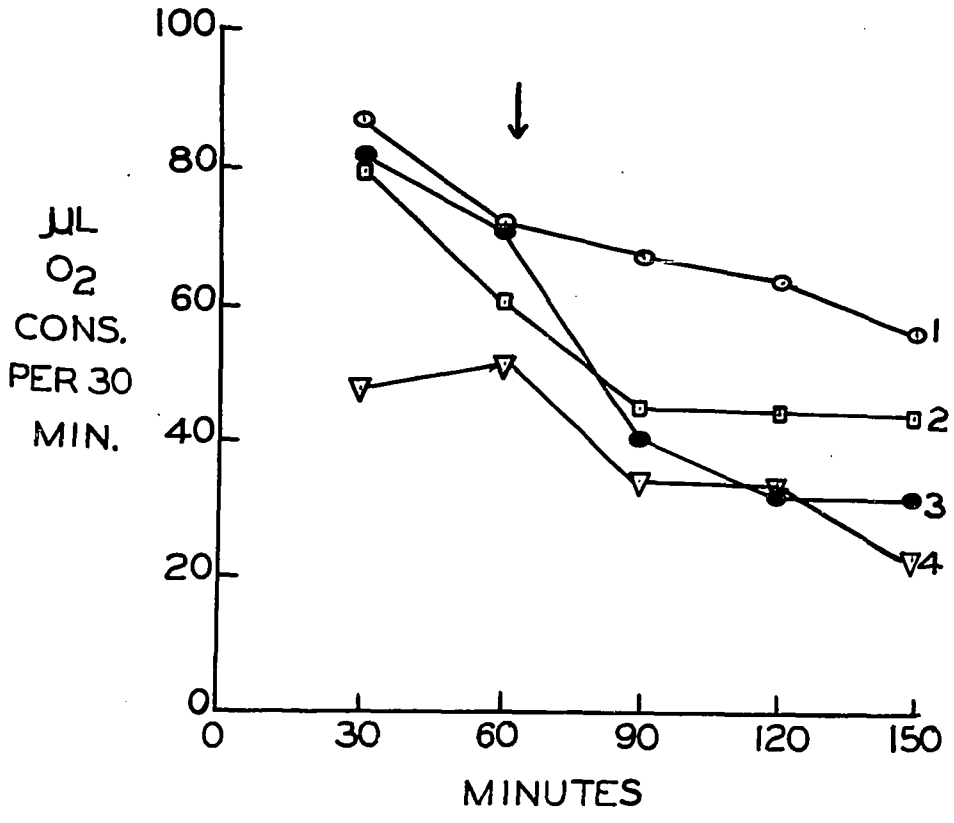

Figure 3. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.05 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. Final concentration of additives: 4: 1 X 10^{-3} M sodium pentobarbital at 0 minutes, 5 X 10^{-4} M ouabain at 62 minutes. 2: 1 X 10^{-3} M sodium pentobarbital at 0 minutes. 3: 5 X 10^{-4}M ouabain at 62 minutes. 1: None.

EFFECT OF OUABAIN ON THE BARBITURATE-DEPRESSED RESPIRATION OF GUINEA PIG KIDNEY CORTEX SLICES

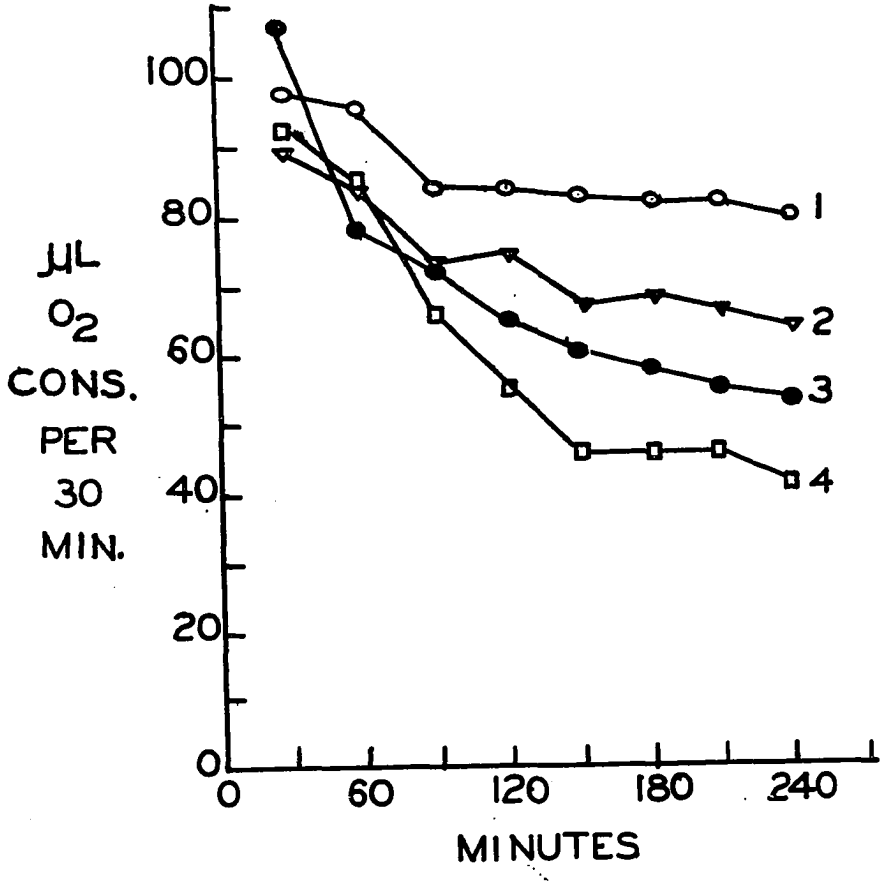

Figure 4. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.06 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 Part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. Final concentration of additives: 1.1×10^{-2} M glucose, all vessels: 4: 1×10^{-3} M sodium pentobarbital at 0 minutes, 5×10^{-4} M ouabain at 120 minutes. 2: 1×10^{-3} M sodium pentobarbital at 0 minutes. 3: 5×10^{-4}M ouabain added at 120 minutes. 1: None.

Pyruvate

Figure 5 represents the results obtained when ouabain (5×10^{-4} M) is added to kidney cortex slices utilizing sodium pyruvate as substrate. In one-half hour, the rate of oxygen consumption is reduced from the control level by an average of 30 percent.

Alpha-ketoglutarate

Figure 6 shows that ouabain, in the same concentration that stimulated the oxygen consumption of heart muscle (5×10^{-4} M) largely prevents the increase in O_2 uptake by kidney cortex slices usually obtained by the addition of 40 micro-moles of alpha-ketoglutarate to the reaction vessels. The average change in rate obtained with this substrate is shown by Curve 1, as compared to Curve 2 which is the rate obtained without the substrate. Curves 3 and 4 show that in the presence of ouabain, the addition of alpha-ketoglutarate causes no upswing in the rate of oxidation. This probably means that the utilization of alpha-ketoglutarate by kidney cortex slices exposed to ouabain at this concentration is almost totally inhibited.

Succinate

Ouabain (5×10^{-4} M) has far less influence on the oxidation of sodium succinate by kidney cortex slices than it has on the oxidation of alpha-ketoglutarate. The succinate oxidation in the presence of ouabain averages only 20 percent lower than the rate obtained from glycoside-free preparations. Figure 7 shows typical results obtained for this set of experiments, indicating only a slight interference with the utilization of this substrate.

EFFECT OF OUABAIN ON THE OXIDATION OF PYRUVATE BY GUINEA PIG KIDNEY CORTEX SLICES

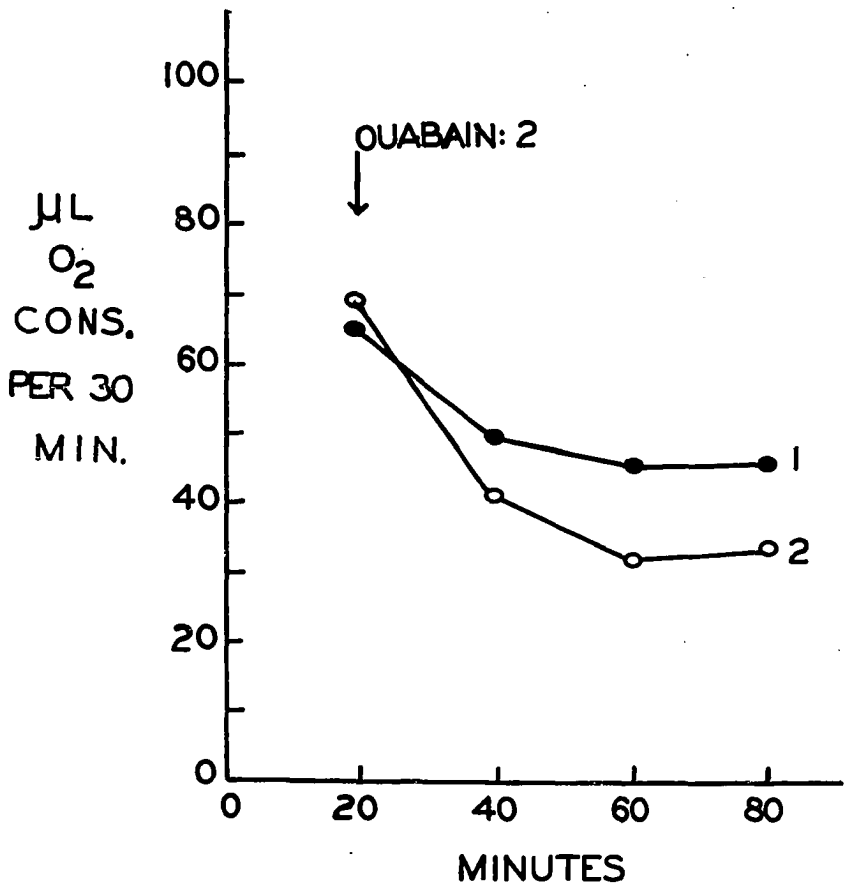

Figure 5. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M Kcl, 4 parts; 0.06 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. Final concentration of additives; Sodium pyruvate, 40 micromoles per vessel. 2: 5×10^{-4} M ouabain added at 22 minutes. 1: None.

EFFECT OF OUABAIN ON THE OXIDATION OF ALPA-KETO-GLUTARATE BY GUINEA PIG KIDNEY CORTEX SLICES

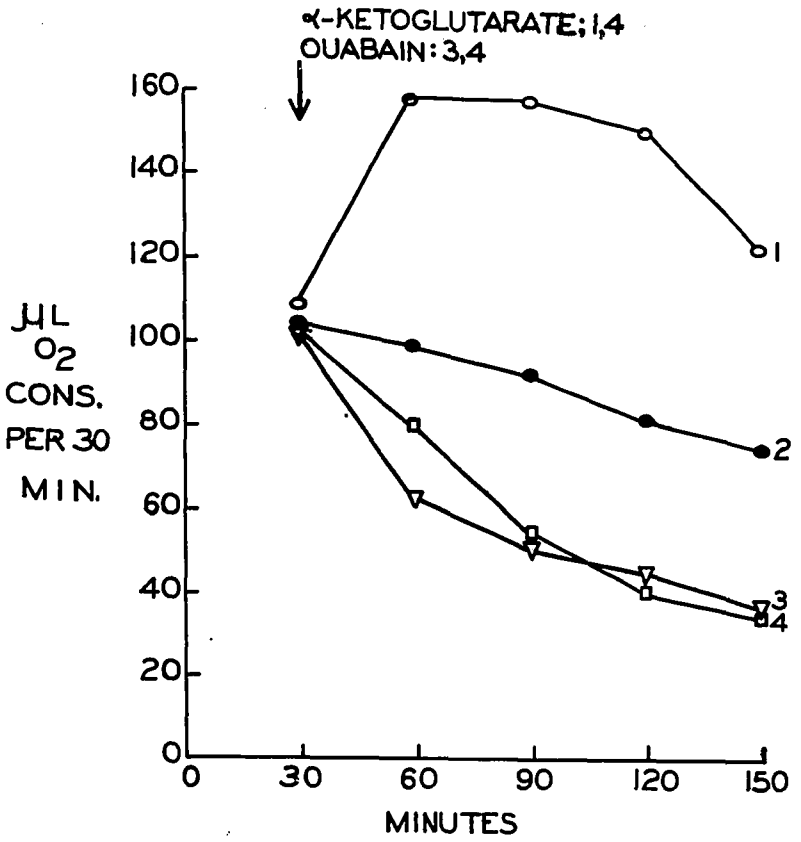

Figure 6. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.06 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. Final concentration of additives: 1: 40 micromoles sodium alpha-ketoglutarate added at 30 minutes. 2: None. 3: 5 X 10^{-4}M ouabain added at 30 minutes. 4: 40 micromoles sodium alpha-ketoglutarate and 5 X 10^{-4}M ouabain added at 30 minutes.

EFFECT OF OUABAIN ON THE OXIDATION OF SUCCINATE BY GUINEA PIG KIDNEY CORTEX SLICES

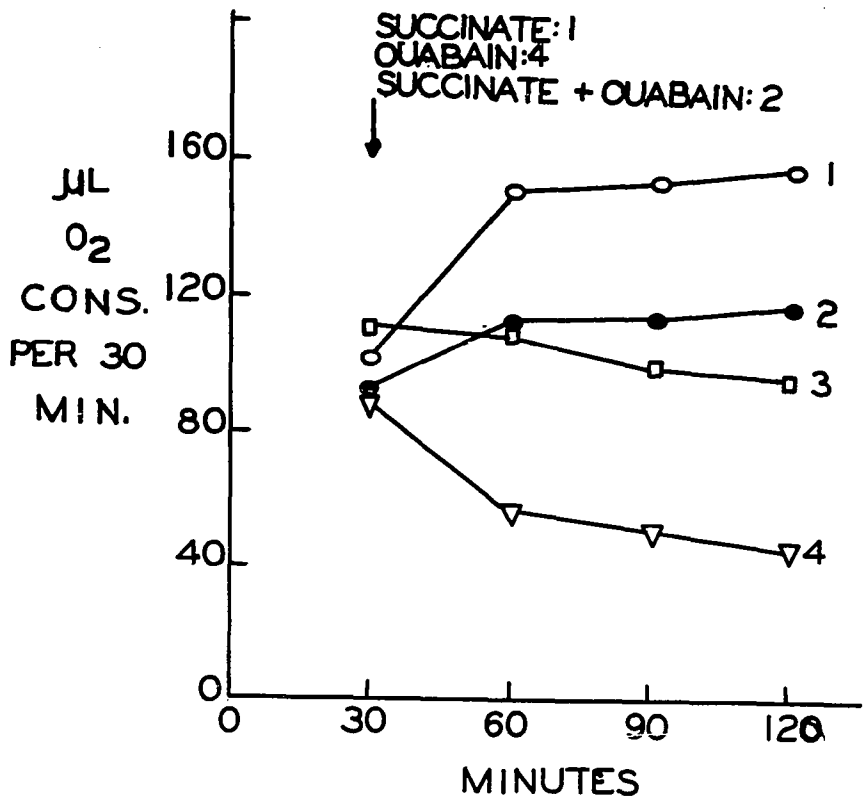

Figure 7. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.06 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. Final concentration of additives: 1: 100 micromoles sodium succinate added at 30 minutes. 2: 100 micromoles sodium succinate and 5×10^{-4} M ouabain added at 30 minutes. 4: 5×10^{-4} M ouabain added at 30 minutes. 3: No additives.

Fumarate and Malate

The inhibition of oxygen consumption caused by the addition of ouabain to kidney cortex slices incubating in a medium containing fumarate or malate is small. The data are inconclusive because the conditions of the experiment were not suitable for the utilization of these substrates. This matter is considered further in the discussion of results.

Effects of Calcium and Potassium Ion Concentrations on the Inhibition of Respiration by Ouabain

In the history of the cardiac glycosides, it was pointed out that calcium ions have a synergistic effect with ouabain regarding cardiotonic activity. When calcium ions are reduced or absent from the physiological medium bathing a heart preparation, the positive inotropic action and stimulation of respiration due to the presence of ouabain are considerably reduced. Addition of calcium ions quickly restores the effect. Conversely, potassium ions diminish the influence of the cardiac glycosides. In the case of the kidney cortex, it was of interest to know if these ions have a function in the inhibition of respiration caused by ouabain. Figure 8 shows the results of experiments in which the effect of ouabain is tested on the oxygen consumption of kidney cortex slices in media with alterations in the concentrations of calcium and potassium. In some vessels, the medium contained one and one-half times the calcium ion concentration that is specified for Krebs-Ringer Phosphate solution with an equivalent reduction in potassium ions (Curves 2 and 5 of Figure 8). The reverse situation was arranged in

EFFECT OF CALCIUM AND POTASSIUM IONS ON THE INHIBITION OF RESPIRATION OF GUINEA PIG KIDNEY CORTEX SLICES BY OUABAIN

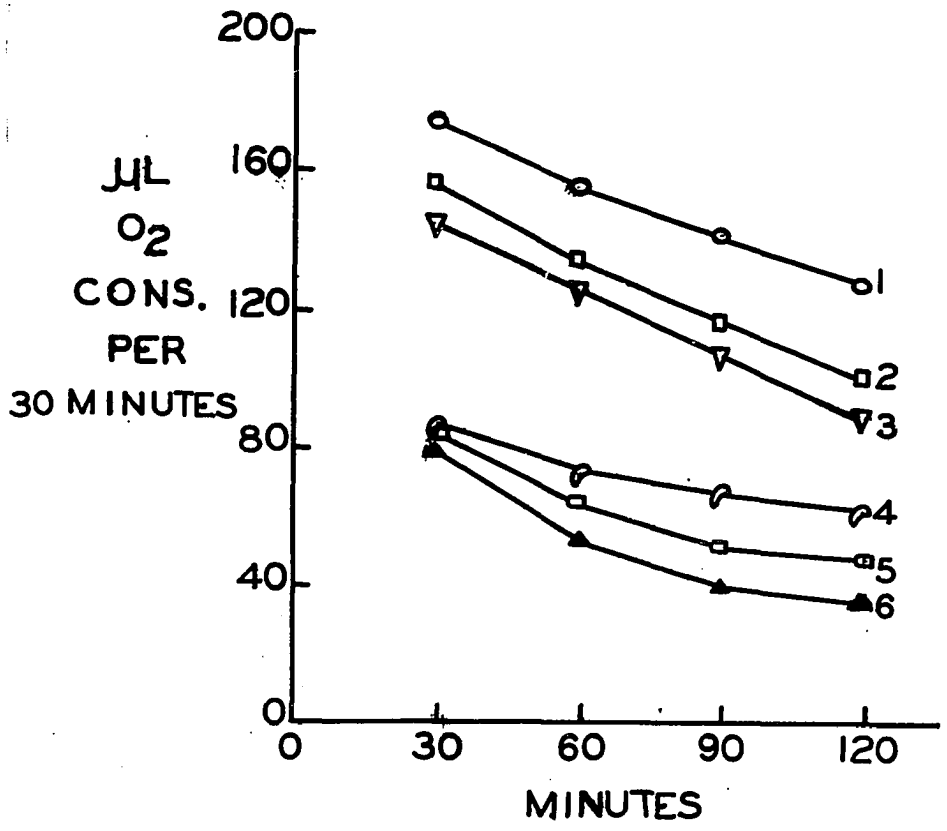

Figure 8. Oxygen consumption per 100 mg tissue (wet). Suspending media: 1 and 4: Krebs-Ringer Phosphate Buffer, pH 7.4. 2 and 5: Krebs-Ringer Phosphate Buffer minus KCl plus an additional 0.44 mM% $CaCl_2$, pH 7.4. 3 and 6: Krebs-Ringer Phosphate Buffer minus $CaCl_2$ plus an additional 45 mM% KCl, pH 7.4. 4,5 and 6: Ouabain 5 X 10^{-4}M at 0 minutes. 1, 2 and 3: No ouabain.

vessels containing excess potassium ions. Alpha-ketoglutarate was employed as substrate since the utilization of this intermediate appears to be the most markedly inhibited by ouabain of those tested. The results disclose that while these imbalances in ion concentration give less oxygen consumption than for physiological medium (Curves 2 and 3 as compared to Curve 1), the proportional decrease in rates of respiration caused by the presence of ouabain is essentially the same in all cases, being approximately 50 percent of the rate of non-glycoside treated slices. The experiments indicate that calcium and potassium ions do not determine the intensity of the inhibition of O_2 uptake caused by 5×10^{-4} M ouabain as it does in cardiac tissue.

Respiration of Kidney Cortex Slices after Toxic In Vivo Doses of Ouabain

These experiments were done as the preliminary step in determining the influence of ouabain on the respiration of kidney cortex after parenteral administration of a lethal dose of the glycoside (total dose 0.3 mg) to adult guinea pigs. About two minutes after injection of the drug, the animals suddenly died. On immediately opening the thorax, the hearts of these animals appeared to be in fibrillation. The kidneys were quickly removed and prepared in the usual way except that the slices were immediately placed into the reaction vessels after slicing in order to prevent possible "leaching-out" of the drug by pooling slices in cold suspending medium until the sectioning was done. Two such experiments have now been done. Figure 9 shows the results obtained. The average rate of respiration of all slice preparations from injected

EFFECT OF A LETHAL DOSE OF OUABAIN ON THE RESPIRATION OF GUINEA PIG KIDNEY CORTEX SLICES

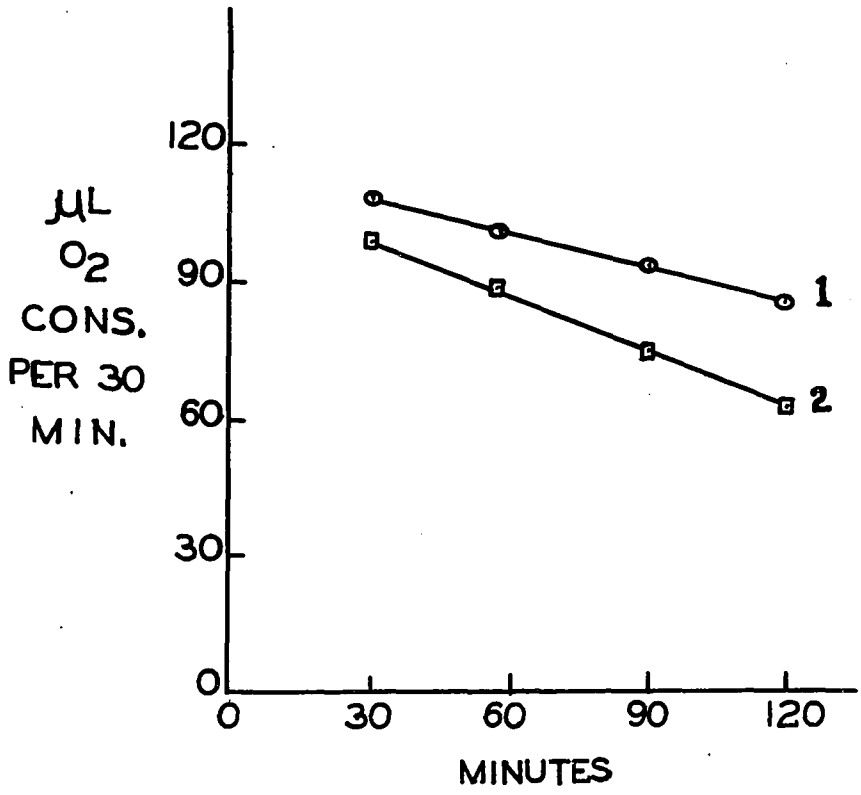

Figure 9. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.11 M $CaCL_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. 1: Animals injected with 0.3 mg ouabain. 2: Control animals.

animals is pictured by Curve 1. Curve 2 is the average for non-injected guinea pigs. All preparations were respiring in the absence of substrate. There appears to be a slight but constant increase in rate of O_2 uptake of kidney cortex tissue from the ouabain poisoned animals. Further investigation will be required to confirm this effect.

Effect of Ouabain on Homogenates of Guinea Pig Kidney Cortex

Figure 10 depicts the typical result obtained with homogenates exposed to ouabain. It has no effect on the rate of oxidation of alpha-ketoglutaric acid. This is in direct contrast to the marked inhibition of ketoglutarate oxidation in kidney cortex slices caused by this drug. Curves 1 and 2, representing vessels oxidizing alpha-keto-glutarate with or without the presence of ouabain, are not significantly different. The effect on endogenous respiration (Curves 3 and 4) is also nil.

EFFECT OF OUABAIN ON THE RESPIRATION OF KIDNEY CORTEX HOMOGENATES

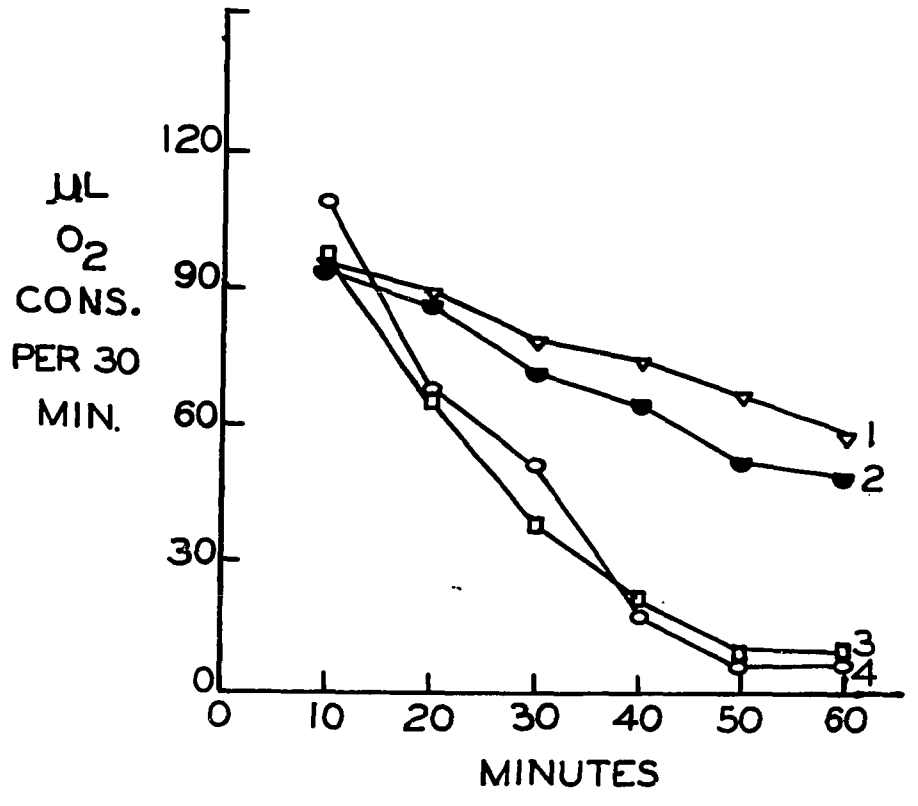

Figure 10. Oxygen consumption per 100 mg tissue (wet). Suspending medium: 0.154 M NaCl, 100 parts; 0.154 M KCl, 4 parts; 0.11 M $CaCl_2$, 3 parts; 0.154 M KH_2PO_4, 1 part; 0.154 M $MgSO_4 \cdot 7H_2O$, 1 part; 0.1 M Phosphate Buffer, pH 7.4, 12 parts. 1: Sodium apha-ketoglutarate, 40 micromoles, ouabain 5×10^{-4} M, 2: Sodium alpha-ketoglutarate, 40 micromoles. 3: 5×10^{-4} M ouabain. 4: None. All preceeding additions made at 0 minutes.

CHAPTER V

DISCUSSION OF EXPERIMENTAL RESULTS

The results point out that while a concentration of 5×10^{-4} M ouabain stimulates the respiration of cardiac muscle (especially of barbiturate-depressed hearts), it interferes with the carbohydrate metabolism of kidney cortex tissue of the guinea pig, with the result that oxygen consumption is reduced. It does not reverse, but, rather, enhances the depression of respiration caused by 1×10^{-3} M sodium pentobarbital. These findings may add some weight to the ascribed specificity of cardiac glycosides for the heart in a therapeutic sense.

The defect in oxidation of carbohydrate by guinea pig kidney cortex slices exposed to ouabain evidently does not involve the glycolytic enzymes, but rather some portion of the tricarboxylic acid cycle. The oxidation of substrates tested were diminished to different degrees. Of these, the oxidation of alpha-ketoglutaric acid was the most severely inhibited by ouabain. A study of the purified enzyme system of alpha-ketoglutaric oxidase would be of value in determining a direct action on the enzyme by ouabain. The much smaller effect of the glycoside on the oxidation of succinate may add support to the hypothesis that the main inhibition occurs in the system that converts alpha-ketoglutarate to succinate. With regard to the oxidation of fumarate and malate, the

results cannot be considered as indicative of the true influence of ouabain on these functions. The inclusion of pyruvate as a source of acetate for condensation with the oxalacetate formed by oxidation of either fumarate or malate would have enhanced their utilization and provided a more accurate picture of the drug effect.

The mechanism of the oxidation of alpha-ketoglutaric acid to succinic acid has been studied (Sanadi and Littlefield, 1952, 1953) and led to this postulated mechanism:

1) $\underset{\text{Alpha-ketoglut. Acid}}{\text{COOH-C(=O)-CH}_2\text{-CH}_2\text{-COOH}} \xrightarrow[Mg^{++}]{\text{Cocarboxylase}} \underset{\text{Succinic Semialdehyde}}{\text{H-C(=O)-CH}_2\text{-CH}_2\text{-COOH}} + CO_2$

2) $\underset{\text{Succinic Semialdehyde}}{\text{H-C(=O)-CH}_2\text{-CH}_2\text{-COOH}} + DPN + CoA\text{-}SH \xrightarrow[Mg^{++},\ \text{Lipoic acid}]{\text{Cocarboxylase}} \underset{\text{Succinyl CoA}}{\text{S-CoA-C(=O)-CH}_2\text{-CH}_2\text{-COOH}} + DPNH + H^+$

3) $\underset{\text{Succinyl CoA}}{\text{S-CoA-C(=O)-CH}_2\text{-CH}_2\text{-COOH}} + PO_4^{\equiv}{}_{\text{inorg.}} + ADP \underset{-H_2O}{\overset{+H_2O}{\rightleftharpoons}} \underset{\text{Succinic acid}}{\text{COOH-CH}_2\text{-CH}_2\text{-COOH}} + CoA\text{-}SH + ATP$

In animal tissues, the decarboxylation reaction (1) has not been separated from the dehydrogenation reaction (2) and the activity has been ascribed to a single enzyme unit which contains cocarboxylase

and lipoic acid. The third reaction, involving the deacylation of succinyl co-enzyme A is not well established but probably follows the overall pattern shown above.

An interesting aspect of this study was the very different results obtained when measuring the effect of the same concentration of ouabain on kidney cortex and heart muscle slices. Since the homogenate studies show that the glycoside does not inhibit the oxidations, the opposite reactions of slices of these two tissues exposed to the drug points to a mechanism which must involve cellular organization linked to at least some part of the tricarboxylic acid cycle, and which is sensitive to extremely small amounts of ouabain. Possibly a ouabain-sensitive metabolite or enzyme existing only in intact cells exist in different concentrations in heart as compared to kidney. An optimal concentration of ouabain may stimulate such a substance in the heart but this concentration may, for kidney cortex, be far in excess, perhaps resulting in a competitive type inhibition of the ouabain sensitive process. A comparative study of these two tissues exposed to varying concentrations of ouabain should verify or exclude this possibility.

By whatever means the drugs act, they are not destroyed in the process, or only very slowly, since the maximum capacity of the body to destroy cardiac glycosides is small, about 50 micrograms per day in the case of digitoxin (Meyer, Friedman, St. George, Bine and Byers, 1952). Little is known concerning the nature of the end-products of the metabolism of glycosides (Okita, 1953).

There does not appear to be any synergism between the effect of ouabain on the respiration of kidney cortex slices and the calcium

ions. This may only mean that calcium ions enhance the effect of cardiac glycosides on the heart by their requirement for contractility of the fibers, on which the glycosides may then act. In any case, it would be very difficult to reconcile the positive inotropic effect of these drugs and the inhibitory effect on kidney cortex respiration to the same biochemical mechanism, and in all probability, these phenomena represent specific differences in response by two different tissues, which may involve widely differing areas of metabolism.

One fundamental similarity has been observed in most of the work done by investigators on cardiac glycosides. This is the absence of any uniform effect on homogenates. In this study, ouabain apparently does not affect the utilization of alpha-ketoglutarate in kidney cortex homogenates, nor oxygen consumption of endogenous preparations. These findings are in agreement with the hypothesis that the structural integrity of the cell is required by cardiac glycosides for their physiological effects, and in view of this, it is doubtful that the inhibition of respiration seen in kidney slices is due to a direct action of ouabain on one or more oxidative enzymes.

If cardiac glycosides could affect the energy metabolism in the intact animal by interfering with the oxidation of carbohydrate, this property might have some bearing on the diuresis occurring during therapy with these drugs, since it is known that energy of respiration is required for the ion selectivity of tubule cells (Whittam and Cavies, 1954).

CHAPTER V

CONCLUSIONS

1. Anaerobic glycolysis of kidney cortex of guinea pig is not altered by 5 X 10^{-4} M ouabain. The lactic acid/CO_2 ratio is identical in all experiments.

2. The stimulatory effect of 5 X 10^{-4} M ouabain on the barbiturate depression of oxygen consumption of cardiac muscle was confirmed.

3. Ouabain, 5 X 10^{-4} M, does not reverse, but rather enhances the barbiturate-depressed oxygen consumption of guinea pig kidney cortex slices. The glycoside itself causes a marked depression of O_2 uptake by kidney cortex slices.

4. The oxidation of alpha-ketoglutaric acid is nearly totally inhibited by 5 X 10^{-4} M ouabain. Pyruvate oxidation is reduced to a lesser degree, while the oxidation of succinate is only 20 percent below the control rate. Results with fumarate and malate oxidation were inconclusive.

5. Calcium and potassium ions, which regulate the intensity of response of heart muscle slices to ouabain, do not possess this property in kidney cortex tissue of guinea pigs.

6. Respiration of guinea pig kidney cortex slices after lethal

in vivo injections of ouabain appears to be elevated. This effect will require further investigation for verification.

7. Ouabain, 5×10^{-4} M, does not influence the endogenous respiration of guinea pig kidney cortex homogenates, nor the oxidation of alpha-ketoglutarate by these homogenates.

CHAPTER VI

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