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CURRENT STATUS OF DRUG RECEPTOR THEORY WITH SPECIFIC REFERENCE TO $Na^+ + K^+$ -ATPase AND OPIATE RECEPTORS

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Abstract

The $Na^+ + K^+$ -ATPase is specifically inhibited by cardiac glycosides and appears to be the inotropic receptor for cardiac glycosides. The interaction of $Na^+ + K^+$ -ATPase with cardiac glycosides shows many characteristics of a good drug receptor model. The binding is specific, of high affinity, is easily measured, and is stable enough to be readily manipulated experimentally. It is possible to show a good correlation between the *in vitro* interaction of cardiac glycosides with the $Na^+ + K^+$ -ATPase and their inotropic effects in perfused hearts. Identification and isolation of the receptor portion of the $Na^+ + K^+$ -ATPase is a more difficult problem and preliminary studies on photo-affinity labelling techniques for $Na^+ + K^+$ -ATPase are reported. Finally, using opiate receptor agonists, a sequence of dose and time response relationships is readily demonstrable in the horse, clearly demonstrating the effects of classic drug-receptor interactions at the clinically relevant level of the racehorse.

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The classical mathematical description of the interaction of a drug with its receptor is expressed in the following notation:

$$D+R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} DR \xrightarrow{k_2} DR \longrightarrow \text{Response}$$

where k_{+1} is the rate constant for formation of the complex, k_{-1} is the rate constant for dissociation (offset) of the complex and k_2 is a proportionality constant which is related to the development of the pharmacological response.

When I started in graduate school at the University of Toronto, some fifteen years ago, the conventional wisdom was that K_{+1} and k_{-1} were extremely rapid events, with half-times in the order of milliseconds and that the direct identification of the drug-receptor complex or the measurement of these time constants would therefore be a very difficult process. Working on my doctoral thesis, however, it became clear to me that I was measuring these constants, k_{+1} and k_{-1}, and that it was quite easy to measure these rates in the cardiac glycoside system, once you got used to the idea. This led me to examine these rates for some other drug-receptor complexes, and this data is presented in Table 1.

Now, the message from Table 1 is relatively straightforward. In it I have listed some well characterized drugs with high affinities for their receptors, and the known rates of association and dissociation of the drugs from their receptors. For three of these drug receptor interactions the time constant for dissociation of the drug from the receptor is in the order of minutes or longer. Given such high drug receptor affinities, with their relatively slow dissociation rates of the drug receptor complex,the study of drug receptor interactions is greatly simplified.

The rule of thumb to be drawn from Table I is that if the affinity of a drug for its receptor is in the order of 10^{-9}M, it is likely that the rates of formation and dissociation of the drug receptor complex will be slow enough to be measured without sophisticated apparatus. This follows from the mathematics of the drug-receptor interaction, for Burgen has shown that the rate of formation of a drug receptor complex is limited by the rate of diffusion of drug molecules in an aqueous environment to less than 2.5×10^{9} liters/second. This rate is an upper limit and it is further likely that only a very small proportion of drug collisions with the receptor

are likely to give rise to the drug receptor complex. This is because the drug will have to strike the receptor in a suitable attitude and configuration, and may have to compete with nonspecifically bound ions and ligands, all of which will tend to reduce the rate of formation of the drug-receptor complex.

Given these circumstances, it is clear that in real life the rate of association of a drug with its receptor will often be a lot less than 2.5×10^9 liters/mole/sec, perhaps easily 100-fold less. Given this or a slower association rate, the dissociation rate for the drug-receptor complex must also be quite slow if the affinity constant (k) is in the order of 10^{-9} M. Given such relatively stable binding and slow dissociation rates, the study of drug receptor interactions may be greatly simplified.

The second phenomenon which occurs when you are dealing with K values in the order of 10^{-9} M is that the amount of drug bound to the receptor is likely to be a significant proportion of the amount of drug in the experimental system. Under these circumstances, it becomes relatively easy to isolate the drug-receptor complex, particularly if the receptor is membrane-bound and the drug highly radiolabelled. In this way simple centrifugation or millipore filtration can be a very effective way of harvesting the drug-receptor complex (Fig 1). Such experimental systems can be fast, easy to work with and flexible, or, in other words, may be very useful experimental systems.

Next, I want to briefly discuss k_2, or the change in configuration of the receptor to the form induced by the drug. By definition the simplest drug receptor interaction is that of an ideal antagonist-receptor interaction. This is because the ideal antagonist is simply a "blocker" which fits perfectly onto the receptor, and does not induce any configurational change in the receptor. Theoretically, and in actual fact, therefore, one often finds that it is the antagonists which have the highest affinities for receptors (Fig 2).

An agonist, however, must by definition induce a change in the configuration of the receptor, and will therefore strain it to some extent. This change will appear as a somewhat lower binding energy for an agonist as compared with an antagonist. Such systems are well known in the opiate receptor system, and lead to the rule of thumb that if you want to start any kind of receptor studies, you should start with an antagonist, as it will likely show the highest affinity and most stable binding (Fig 2).

The last concept that I want to leave you with before we start to look at specific receptor systems is the concept of a chemically induced change in a receptor. The first system in which I encountered such changes was in the phosphorylase b-phosphorylase a transformations, and in the Na^+K^+-ATPase, where they are the key to understanding the pumping activity of this enzyme. The basic idea here is that while ligands such as drugs or ions can induce configurational changes in receptors, equivalent changes can be induced by chemically modifying the receptor itself such as by phosphorylating it under some circumstances (Fig 2).

The specific receptor system that I am going to discuss today is the Na^+K^+-ATPase, which is now considered to be the pharmacological receptor for the cardiac glycosides. The Na^+K^+-ATPase is the enzymatic basis for the sodium pump, whose existence was first postulated in 1941. A stoichiometric relationship between Na^+ and K^+ transport was first proposed by Harris and Maizels in 1951, demonstrated by Glynn in 1956 and measured by Post and Jolly in 1957. Thus, by the mid-nineteen fifties it had become evident that there was an active, linked transport of Na^+ and K^+ across the cell membranes and the next step was to identify the source of energy for this transport.

From a consideration of the energy sources available in both red cells and other tissues, it was suggested that ATP was a possible energy source for this transport. By the mid-nineteen-fifties, experiments in squid axons had shown that ATP was very likely the prominent energy source for the sodium pump, and a number of investigators noted that this suggested that the ATPases of cell membranes would bear closer investigation.

This idea was picked up by Jens Skou in Denmark, who did not have squid giant axons readily available to him. He therefore obtained the closest approximation of squid axons readily available to him, which were crab leg nerves. He got the crabs from the local Baltic fishermen, dissected out their leg nerves, and made microsomal membrane preparations from them. The remainder of the crabs were boiled and eaten in the department. His department chairman complained about the smell of boiling crabs, but was assured that it was an essential part of very important work. These crabs must have been very good eating for Jens Skou, for as well as annoying his department chairman, they made him famous.

Using his membrane preparation, Skou was readily able to show the presence in it of an Mg^{++} activated ATPase that required both sodium and potassium ion for full activity (Fig 3). This was a very unusual combination of ions to activate an ATPase, and Skou was also able to show that the kinetics of the activation by these ions more or less paralleled what one might expect of an ATPase that was part of a sodium pump. At first nobody paid very much attention to this strange ATPase, until Skou met Bob Post, who had been working on cation transport in red cells.

When Post met Skou face to face, it immediately became apparent to him that Skou had an experimental grip on something exciting. His first question was, "Does ouabain inhibit the ATPase?" When Post had explained that ouabain is the experimentalists cardiac glycoside, and that cardiac glycosides as a class specifically inhibit active sodium transport, the purpose of his question was clear. Together they telephoned Aarhus and the first $Na^+ + K^+$-ATPase cardiac glycoside experiment was designed and directed over the telephone. Ouabain, of course, stopped Skou's $Na^+ + K^+$-ATPase preparation dead in its tracks, sometimes toward the end of the nineteen fifties.

In the mid-sixties, I came into this picture as a graduate student at the University of Toronto. By this time, high specific activity tritiated ouabain had become available, and the basic reaction sequences of the pump was beginning to be elucidated. For my doctoral thesis, Amar Sen and I started a detailed study of the binding of cardiac glycosides to the $Na^+ + K^+$-ATPase.

Before I can get into the mechanism by which the cardiac glycosides inhibit the pump, I first have to tell you a little about how the pump works. The orientation and stoichiometry of the pump are shown in Fig 4. The pump binds ATP, Na^+ and Mg^{++} at the inner surface of the plasma membrane. During the first portion of the transport cycle the 3 Na^+ ions are pumped out of the cell and the products of the reaction cycle, ADP and Pi are released inside the cell. During the second portion of the transport cycle the pump picks up two K^+ ions from outside the cell and pumps them into the cell. The pump is blocked by ouabain, acting only from outside the cell.

An abbreviated reaction cycle for the enzyme is shown in Fig 5. In the presence of Na^+ and Mg^{++} the enzyme is phosphorylated from ATP, to yield E_1-P and ADP. Then the phosphorylation of the enzyme energizes a configurational change in the enzyme and it goes through the first stage in the pumping cycle, flipping from E_1-P to E_2-P. At this stage the Na^+ is pumped, and the phospho-enzyme becomes unreactive with ADP. In the E_2-P form it becomes reactive with K^+, and, as we will show you in some detail, with ouabain.

It turns out that although ouabain is a very potent inhibitor of the $Na^+ + K^+$-ATPase, with a very high (10^{-8} M) affinity for the enzyme, it does not bind at all to the native enzyme. If you take 100 μg of enzyme, put them into a test tube, add ouabain, you can hardly demonstrate any "specific" binding of glycoside to the enzyme. If you simply add a little Mg^{++} to the system you will find an increase in it, and if you add Mg^{++} plus Pi you will find a lot of binding (Fig 6). Now, the really interesting thing happens if you add sodium ion to the system.

It turns out that sodium ion inhibits the Mg^{++} or Pi stimulated pattern of binding to the $Na^+ + K^+$-ATPase. If you add sodium it inhibits the binding and if you add it to a system in which the glycoside is already bound it displaces the ouabain (Fig 6). The kinetics of this sodium interaction are interesting.

As shown in Fig 7, if the concentrations of ouabain, Mg^{++}, and particularly Pi in the system are low, sodium ion readily inhibits the binding. Low concentrations of sodium are sufficient to do it, and the kinetics of the inhibition are hyperbolic. If, however, the concentrations of Pi and ouabain in the system are high, then kinetics of the sodium inhibition become sigmoidal. In other words, Mg^{++}, Pi and ouabain are ligands which favor a different conformation of the enzyme different from that favored by Na^+, and in their presence Na^+ has to cooperate to bind to the enzyme and displace ouabain from the enzyme. This cooperativity in the sodium ion inhibition of the enzyme suggests that sodium ion "draws" the enzyme into an E_1 type configuration and stabilizes the enzyme in that configuration. In this configuration the enzyme is "loaded" with sodium, and ready to enter an active transport cycle (Fig 7).

When the enzyme is in the presence of sodium, you have to give it what appears to be a high energy phosphate bond to get it to bind ouabain. For example, Fig 8 shows the time course of binding of ouabain to the $Na^+ + K^+$-ATPase in the presence of sodium magnesium and different nucleotides. Binding starts when you add the nucleotide, peaks within minutes, and then decays away to background. When you perform the experiment in this way, binding is transient, in exactly the same way as the phospho-enzyme is. My interpretation of these events was that the nucleotide was phosphorylating the enzyme and that ouabain was binding to the phospho-enzyme. Others, however, were of the opinion that the ATP stimulated binding could be explained by assuming that binding of ATP to the enzyme was alone sufficient to produce a configurational change in the enzyme and binding of the ouabain. These different hypotheses are shown in the scheme of Fig 9. To settle this argument, I did the experiment of Fig 10.

The experiment of Fig 10 was made possible by the availability of β-γ methylene ATP. β-γ methylene ATP is an analogue of ATP in which β & γ phosphates are bonded by a methylene group instead of by an oxygen bridge. β-γ methylene ATP is therefore an analogue of ATP which may be expected to bind to the enzyme, produce the configurational changes associated with the binding of ATP, but not be able to phosphorylate the enzyme. For this reason the effects of β-γ methylene ATP in an appropriate experimental system might allow us to choose between the two hypotheses outlined above.

To set up the experiment to distinguish between these two hypotheses, I allowed the enzymes to bind ouabain in the presence of Na^+, Mg^{++} and Pi. I then challenged the system by adding either ATP or β-γ methylene ATP to the system. As shown in Fig 10, β-γ methylene promptly and completely inhibited {^{3}H}-ouabain binding to the system, while ATP itself stimulated binding. These responses are consistent with my hypothesis that it is the phosphorylating actions of ATP which stimulate {^{3}H}-ouabain binding to this enzyme in the presence of Na^+, and that the simple binding of ATP inhibits ouabain binding.

Having determined how ouabain binds to the Na^++K^+-ATPase in vivo, we next elected to study the stability of the enzyme-ouabain complex. These experiments were prompted by findings in other laboratories which suggested that the interaction of cardiac glycosides with the Na^++K^+-ATPase was irreversible, while in our hands the reaction was clearly reversible at 37°. We started these experiments by binding {^{3}H}-ouabain to the guinea pig kidney enzyme and then allowing it to dissociate at different temperatures (Fig 11). The experiment clearly shows that the stability of the ouabain enzyme complex is temperature-dependent, that at 37° the ouabain-guinea pig enzyme complex is quite reversible, but that at 0° the complex is quite stable. This stability of the enzyme glycoside complex at 0° is very important, because it means that one can form the drug-receptor complex at 37°, then hold it bound to the enzyme for preparative or other purposes at 0°, and then warm it up to 37° for further studies of the enzyme-glycoside complex.

The reversibility of the guinea pig kidney enzyme-ouabain complex at 37°, compared with its relative stability at 37° in other species, led us further to examine the stability of the enzyme cardiac glycoside complex in enzyme preparations from a number of different species. Figure 12 shows the stability of these enzyme-ouabain complexes in a number of different tissues. As can be seen, the stability of the complex varies from being relatively unstable in the guinea pig heart enzyme to being quite stable in the dog kidney preparation. There are, therefore, substantial species-dependent differences between the stabilities of these

complexes from species to species, and it appears that one can account for the well known species dependent differences sensitivity to cardiac glycosides by virtue of the differences in the dissociation rates alone.

One of the things that we noticed at this point was that the rate of dissociation of ouabain from guinea pig kidney $Na^+ + K^+$-ATPase was about the same as the rate of loss of the inotropic effect in perfused guinea pig hearts. In other words, if you "hang" a guinea pig heart on a Langendorff apparatus, perfuse it with ouabain to produce an inotropic effect, and then let the inotropic effect decay, the rate at which the effect decays is about the same as the rate at which ouabain is known to dissociate from the enzyme. This, however, is just what one would expect if the rate of dissociation of the glucoside from the enzyme was rate limiting for the offset of the cardiotonic effect of these drugs. Of course, if this hypothesis is true, then the rates of offset of the cardiotonic effects of ouabain in other species should parallel their much slower rates of dissociation from the $Na^+ + K^+$-ATPase.

Figure 13 shows the rates of dissociation of $\{^3H\}$-ouabain from different cardiac $Na^+ + K^+$-ATPase preparations. The rates differ between the species, the dissociation being relatively rapid from guinea pig heart enzyme and slow from dog heart enzyme. Similarly, the rates of offset of the inotropic effects in these species (Fig 14) approximately parallel the rates of dissociation of ouabain from the $Na^+ + K^+$-ATPase (Fig 15), further supporting the hypothesis that the $Na^+ + K^+$-ATPase is the inotropic receptor for the cardiac glycosides.

In a further series of experiments, my colleague, Tai Akera, studied the relationship between the rate of onset and offset of inotropy and the binding of glycosides to the $Na^+ + K^+$-ATPase. In these experiments he perfused isolated guinea pig hearts with ouabain and monitored the time course of onset and offset of the inotropic effect (Fig 16). Then the hearts were cut down from the Langendorff apparatus, cooled to 0° and the initial rate of $\{^3H\}$ ouabain binding to the enzyme measured. Since the enzyme glycoside interaction is relatively stable at 0°, he could estimate the amount of glycoside bound to the enzyme by the inhibition of the initial rate of binding of $\{^3H\}$-ouabain. These data are shown in Fig 17, where the relationship between the onset of ouabain inotropy and dissociation and occupancy of the $Na^+ + K^+$-ATPase is demonstrated.

Similarly, if one takes slices of tissue from these hearts and studies the initial rate of uptake of Rb^{86}, one finds the pumping activity

being reduced as the inotropic activity increases and then returning to control (Fig 18). The experiments suggests that occupancy of the enzyme is associated with inhibition of Rb^{86} uptake showing that the cardiotonic effect of the glycosides is also associated with the inhibition of ion transport by the cardiac tissue.

Finally, to identify the cardiotonic steroid binding site on $Na^+ + K^+$-ATPase, an irreversibly binding cardiac glycoside is needed. Attempts to irreversibly label this site with alkylating agents were unsuccessful. A variation to this approach and a solution to the problem of low receptor reactivity is photoaffinity labeling. In photoaffinity labeling the label is photoactivated after it is specifically bound to the active site and such a photoactive label can readily insert onto carbon-hydrogen bonds. To this end we synthesized 3-acidoacetylstrophanthidin and used it to irreversibly inhibit $Na^+ + K^+$-ATPase, presumably by interacting at the cardiotonic steroid binding site.

The structure of 3-azidoacetylstrophanthidin (AAS) is shown in Fig 19. In the presence of ultraviolet light of appropriate wavelength the double bond of the acidoacetyl group is activated to form a highly reactive intermediate which can insert into carbon-hydrogen bonds. The inhibition of rat brain $Na^+ + K^+$-ATPase by different concentrations of AAS or strophanthidin is shown in Fig 20. The data show that both agents are approximately equipotent inhibitors of rat brain $Na^+ + K^+$-ATPase and indicate that the apparent affinity of this enzyme for AAS is in the order of 1×10^{-6} M or less. The experiment suggests that AAS may bind to the cardiotonic steroid binding site of $Na^+ + K^+$-ATPase. The data of Fig 21 show that AAS also increases the contractile force of isolated guinea pig atria. The experiment provides direct evidence for the ability of AAS to interact with cardiotonic sites in these atrial strips.

Table II shows the results obtained when this enzyme was incubated with AAS in the presence and absence of acetylphosphate. The mixture was exposed to UV light at room temperature or stored for equivalent time periods at room temperature shielded from the light. In this experiment the enzyme activity observed after washing the samples shielded from UV light in the absence of acetylphosphate was set at 100%. If the enzyme was incubated in presence of AAS but not exposed to UV light and then centrifuged and resuspended, approximately 5% inhibition of enzyme activity was observed. Similar losses in enzyme activity were observed when the enzyme was incubated with acetylphosphate with or without UV light exposure, or with AAS plus acetylphosphate without UV light exposure. However, if the enzyme

was incubated with with AAS, and acetylphosphate, and then exposed to UV light, the loss of enzyme activity increased almost 3-fold. Since this is the only condition under which photoactivation of AAS specifically bound at the CS binding sites of this enzyme is expected to occur, the data suggest that AAS is an effective cardiotonic steroid site-directed photoactivated inhibitor of $Na^+ + K^+$-ATPase.

Although the increment of irreversible inhibition obtained by photoactivation was small, its magnitude is consistent with the experience of others. Ruoho and Kyte (1974) using C^1-cymarin derivatives observed irreversible binding of only 2% of the total number of counts added to $Na^+ + K^+$-ATPase preparations. Similarly, Hixon and Hixon (1973) observed covalent binding of only 7% of their labeled 3-azidopyridine nucleotide to NAD-dependent dehydrogenase. These lower levels of incorporation are typical of photoaffinity experiments, in which only a fraction of the label is actually photo-activated and a considerable proportion of the molecules photo-activated enter into side reactions with water.

Because the reaction conditions under which irreversible inhibition of this enzyme by AAS was obtained are relatively mild, it should be possible to increase the amount of inhibition obtained by careful selection of UV wave lengths, intensity and duration of irradiation, and drug concentrations. Since the UV exposure itself used in these experiments caused little inactivation of the enzyme, it should be possible to correlate the incorporation of labeled AAS into the CS binding sites of this enzyme with irreversible inhibition. In this way AAS may prove a useful tool to further investigate the suggestions of Ruoho and Kyte (1974) that the large polypeptide subunit of $Na^+ + K^+$-ATPase contains at least part of the cardiotonic steroid binding sites of this enzyme.

We now switch from the level of the receptor to the level of the whole animal to show the behavioral results of a drug receptor interaction and how clearcut they can be at the level of the racehorse. Although the narcotic analgesics have long been used in horses, there are few descriptions of their actions in horses. A recently synthesized, highly potent narcotic analgesic called fentanyl is reportedly widely used in racing horses in the United States, but little data as to its effects on the horse exist. Standard textbooks of veterinary pharmacology have little information to offer on the action of narcotic analgesics *per se* in the horse.

In the human, fentanyl is a potent narcotic analgesic with a rapid onset and short duration of action. It has a profile of pharmacological activity similar to that of morphine, except that it does not cause

emesis and histamine release. It is considered to be about 150 times more potent than morphine. After IV injection in man, peak analgesia appears after about three to five minutes and lasts for 60 minutes. Fentanyl is a Schedule II controlled substance in the United States.

During the course of our work with fentanyl, it became clear that fentanyl is a potent behavioral stimulant in the horse. Fentanyl acts to induce a very clear-cut and specific locomotor response in the horse, and this activity lends itself to quantitation simply by counting the number of steps the animal takes in a 2-minute period. This ability of fentanyl to stimulate locomotor activity is apparently common to all narcotic analgesics, and can be readily demonstrated in the horse with a minimum of equipment.

In a typical experiment, Thoroughbred or Standardbred horses between 4 and 18 years of age and 400 to 500 kg body weight were used. The animals were kept at pasture and brought into specially shielded box stalls at least 24 hours prior to each experiment. The box stalls measured 13¼ feet square and had straw-covered earthen floors. The bulk of the work reported here was performed in these stalls, with plywood sheeting on the box grillwork to reduce interaction with horses in other stalls. A 12" x 12" glass window in the plywood shielding was used for observation.

During the experiment period the horse was scored for one event if it lifted its left foreleg and took a step. Movements of the left leg not resulting in relocation of the foot, such as scratching or pawing, were not counted. To assist the observer in tracking these events the left front leg of the horse was wrapped in white tape. Each event was tallied on a hand counter and the cumulative score logged every two minutes.

This scoring method allows accurate characterization of the response of individual horses to narcotic drugs. Figure 22 shows typical results which were obtained when a single horse was dosed with normal saline and increasing doses of fentanyl. On saline, the spontaneous locomotor activity of this horse was usually about 4 steps/2-minute period except for immediately after the IV injection, and this pattern was consistent over the one-hour control period. The lowest dose of fentanyl, 0.001 mg/kg or approximately 0.5 mg to a 1,000 lb horse, produced no observable behavioral effects. A 5-fold increase in dose produced a small (30 steps/2-minute period) increase in locomotor activity which peaked at four minutes and then declined toward the control level. Similarly increasing doses produced further increases in both the rate and duration of the response, the effect peaking at about 110 steps/2-minute period

and then decaying away. In this particular horse, no differences in response between the two highest doses tested were seen.

Figures 23 and 24 show that essentially similar responses were obtained after etorphine and meperidine. In each case the response peaked between 60 and 120 steps/2-minute period. Etorphine, however, was remarkably potent, producing maximum activity at a dose of 0.00040 mg/kg, while meperidine was much less potent, requiring 5 mg/kg for a maximal response. Similar responses were seen with a wide range of other narcotic analgesics.

Figure 25 shows peak locomotor responses for each dose of these narcotic analgesics and apomorphine from Tobin et al plotted as log dose-response curves to allow direct comparison of the potency and efficacy as locomotor stimulants of the various members of this group of drugs.

The curves for all the full agonists tested are remarkably parallel. The curve for the only non-narcotic included, apomorphine, has a much steeper slope, indicating a different mechanism of action, i.e. apomorphine does not bind to the opiate receptor. The two partial agonists investigated, pentazocine and butorphanol (the latter is not included in the illustration), produced very shallow dose response curves. The rank order of the drugs in inducing locomotor activity in the horse parallels both the ordered ranking of the capacity of the same drugs to induce analgesia in man (DiPalma, Hoover). Furthermore, there is also good agreement between this family of locomotor response curves and the apparent affinity of each drug for the opiate receptor (Pert & Snyder).

The schematic seen in Fig 26 represents the most apparent behaviors of the horse associated with the narcotic analgesics. When a small amount of drug was administered or under other circumstances when locomotor activity rates were low, the horses consumed large amounts of hay (water buckets were removed from the stalls before each experiment was begun so no information is available on water consumption). At higher rates of locomotor activity, the horses often snatched bites of hay from the wall rack. For all the narcotics tested, incoordination determined the maximum dose administered. On the other hand, horses receiving apomorphine remained coordinated at all dose levels, with maximum activity apparently determined by the physical limitation of the stall size.

TABLE I

DRUG RECEPTOR COMPLEX	K	k_{+1}	k_{-1}
Atropine-muscarinic receptor	1×10^{9}	1.7×10^{6}	1.7×10^{-3}
Sulfonamide - Carbonic Anhydrase	1×10^{8}	2×10^{7}	0.2
$Na^{+}+K^{+}$-ATPase-Ouabain	4×10^{7}	1×10^{5}	4×10^{-2}
Aminopterin-Folate Reductase	1×10^{11}	2.5×10^{9}*	?

K = Affinity constant

k_{+1} Liters/moles/second

k_{-1} Sec^{-1}

*Theoretical maximum association rate.

TABLE 11

Photoactivated inhibition of rat brain $Na^+ + K^+$-ATPase by 3-azidoacetylstrophanthidin.

About 200 µg of rat brain $Na^+ + K^+$-ATPase were incubated with 100 mM Na^+, 5 mM Mg^{++} and 0.5×10^{-6} M AAS. Specific binding of AAS to the enzymes was supported by the addition of 1 mM acetyl phosphate which was added 15 min before the start of ultraviolet exposure, Ultraviolet exposure was for 15 min at 23° as described in METHODS. After ultraviolet exposure samples were centrifuged at 40,000 g for 10 min and resuspended in 50 mM Tris buffer, pH 74. The centrifugation and resuspension was repeated and $Na^+ + K^+$-ATPase activity assayed. Enzyme activity is expressed as a percentage, ± standard error, of $Na^+ + K^+$-ATPase activity observed in enzymes not exposed to AAS or ultraviolet light which averaged 270.5 ± 15.3 moles Pi/mg protein/hr.

Substrates	UV light	Mean activity ± S.E.M.
AAS + ACP	+	84.3 ± 3.9[a]
AAS + ACP	-	95.0 ± 1.8[b]
ACP	+	95.4 ± 1.0
ACP	-	97.2 ± 2.4
AAS	+	94.3 ± 1.4
AAS	-	100.00±0.00

[a]Significantly different from b, $p < 0.05$.

Fig 1 Proportions of drug-Receptor Complex and Free Drug in Low and High Affinity Drug Receptor Systems.

The upper panel represents a typical high affinity drug receptor system, where the number of drug molecules in the system is due to the number of receptor molecules in the system. Under these circumstances, the amount of receptor bound drug (RD) is a substantial proportion of the total amount of drug and is relatively easy to identify. For example, if the RD complexes can be precipitated by centrifugation, the "button" will contain 4 RD complexes for every free drug molecule (D).

On the other hand, in a low affinity system the amount of bound drug (RD) is small, and the amount of free drug is large. Under these circumstances, the amount of bound drug is a very small proportion of the total amount of drug present, and centrifugation to harvest the bound drug may not be able to demonstrate discernible amounts of drug receptor complex because of the large amounts of free drug present.

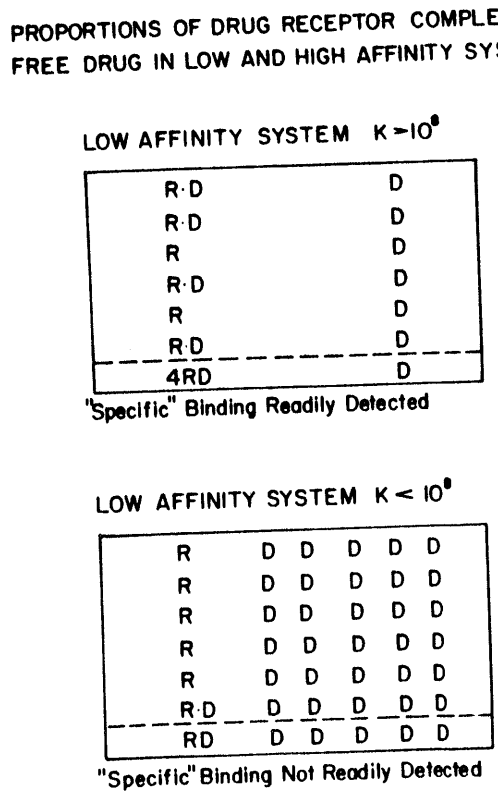
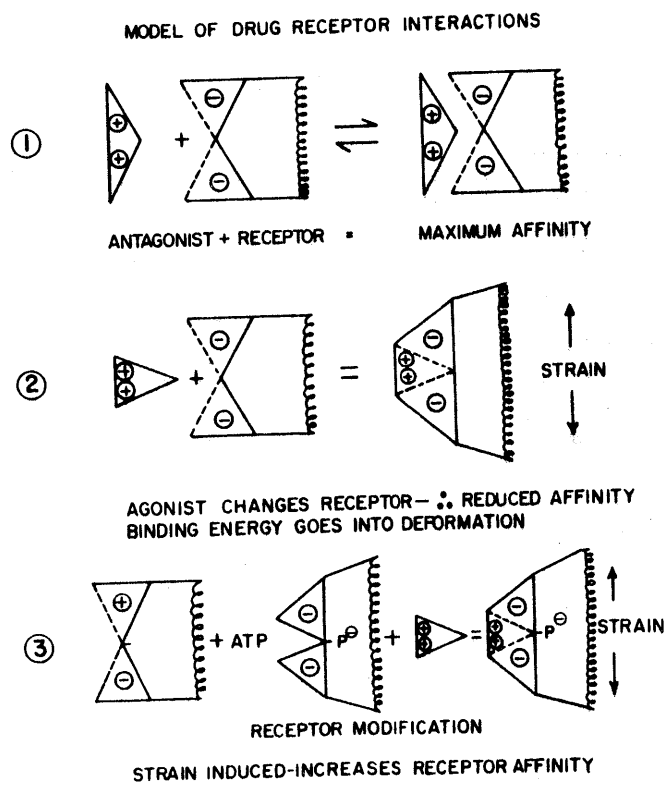


Fig 2 Model of Drug-Receptor Interactions.

In this representation, the drug receptor is presented as a hinged structure, kept in its relaxed configuration by a spring. When this structure interacts with an antagonist (upper panel, 1), the fit is perfect, of high affinity and no deformation of the structure takes place. When the receptor interacts with an agonist (middle panel, 2), deformation of the receptor takes place. The energy that goes into this deformation is indicated by the strain on the srping, and appears as a reduced affinity of agonists for their receptors. It is also possible (3) to have a system whereby phosphorylation changes the receptor configuration and thus increases the affinity of the drug for its receptor.



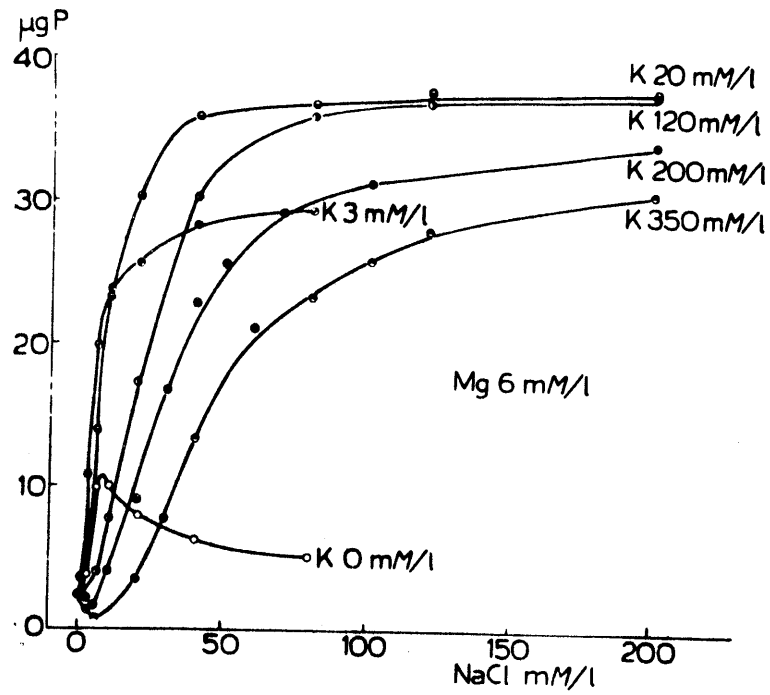


Fig 3 Enzyme activity in relation to the concentration of Na^+ in the presence of Mg^{++}, 6 mM/l, and different concentrations of K^+. Abscissa, sodium concentration in mM/l; ordinate, µg P removed from ATP in 30 minutes.

(Reproduced with permission from Skou, Biochim Biophys Acta, 1957).

Fig 4 Stoichiometry and localization of the sodium and potassium ion pump in human erythrocytes. *Figure reprinted by permission from Regulatory Functions of Biological Membranes, ELsevier Publishing Co., Amsterdam, 1968.*

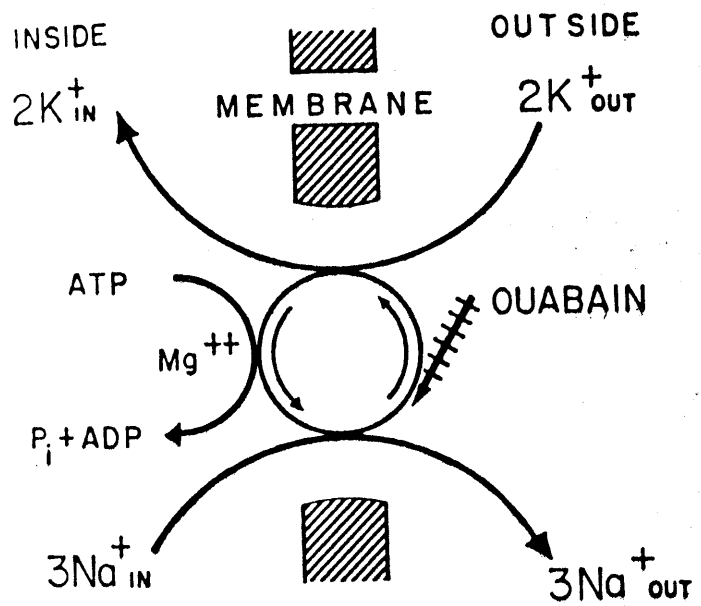


Fig 5 A partial sequence for $(Na^+ + K^+)$-ATPase as proposed by Albers and coworkers. "E" indicates the enzyme and "E_1-P" and "E_2-P" indicate two phosphorylated intermediates. "NEM" indicates a step which can be selectively inhibited by N-ethylmaleimide or by oligomycin. *Figure reprinted by permission from J. Biol. Chem., 1966, 241:1882.*

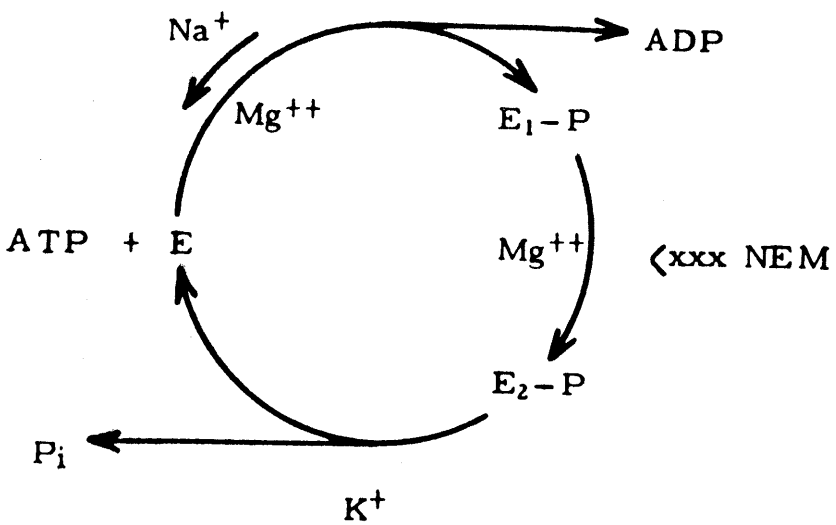


Fig. 6 Time course of {^{3}H} ouabain binding by the Na^+ -inhibited pathway. The reaction tubes contained enzyme, 10 mM imidazole-glycylglycine and 2.5 10^{-7} M {^{3}H} ouabain. After the indicated times at 37° binding was stopped by centrifugation as described under MATERIALS AND METHODS. Further additions were as below. ■-■, binding in the presence of 1 mM Mg^{2+} and 1 mM P_i; □-□, binding in this medium with 60 nM Na^+ added; O-O binding in the presence of 1 mM Mg^{2+}; ●-●, binding in the presence of 1 mM Mg^{2+} 1mM K^+; X, binding in the presence of 1 mM Mg^{2+} plus 16 mM K^+. (Reproduced with permission from Tobin and Sen, Biochem Biophys Acta, 1970).

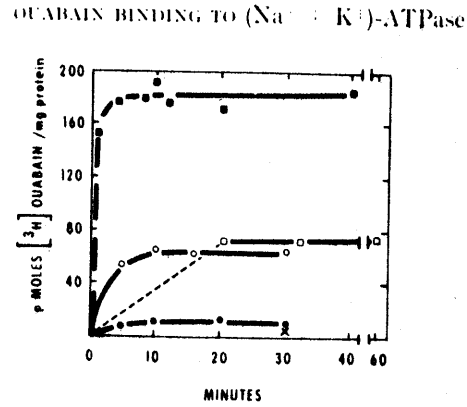


Fig. 7 Na^+ inhibition of ouabain binding: effect of varying the antagonistic ligands. { ^3H} ouabain was allowed to bind to the enzyme under different conditions for 20 minutes. □-□ indicates ouabain binding in the presence of 0.1 mM Mg^{2+} and 2.5 10^{-7} M ouabain, 4 mM Mg^{2+}, 1 mM P_i and 2.5 10^{-7} (0-0), 2.5 10^{-6} M (■-■), or 2.5 10^{-5} M (●-●) ouabain with Na^+ varied as indicated. Binding is plotted as percent of the value at 0.0 mM NaCl. The inset shows a Hill plot of the same data. (Reproduced with permission from Tobin and Sen, Biochim Biophys Acta, 1970).

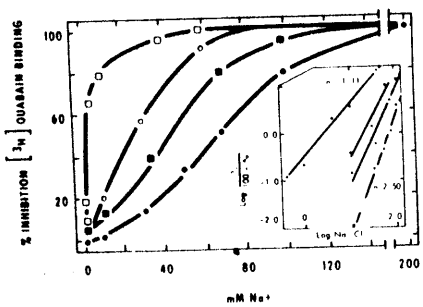


Fig 8

Time course of [γ-^{32}P] ATP$_3$-dependent labelling and nucleotide-supported ^{3}H -ouabain binding to $Na^+ + K^+$-ATPase. (A) Guinea-pig kidney enzymes were incubated with 100 mM Na^+ and 5 mM Mg^{2+} at 37° C. At indicated zero time 25 µM [γ-^{32}P] ATP was added to start the labelling reaction. The reaction was stopped with trichloroacetic acid. (■—■) shows the amount of ^{32}P-labelling of the enzyme at the indicated time points. Labelling is expressed as a percentage of peak labelling for each of four different experiments which averaged 122 pmol ^{32}P/mg protein. (B) At indicated zero time the binding reaction was started by the addition of 50 µM of each of the indicated nucleotides and stopped by centrifugation at the indicated time points. The symbols show the binding of [^{3}H]-ouabain supported by each of the nucleotides with no deductions made for background labelling.

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Ouabain, Cassaine and the $Na^+ + K^+$-ATPase

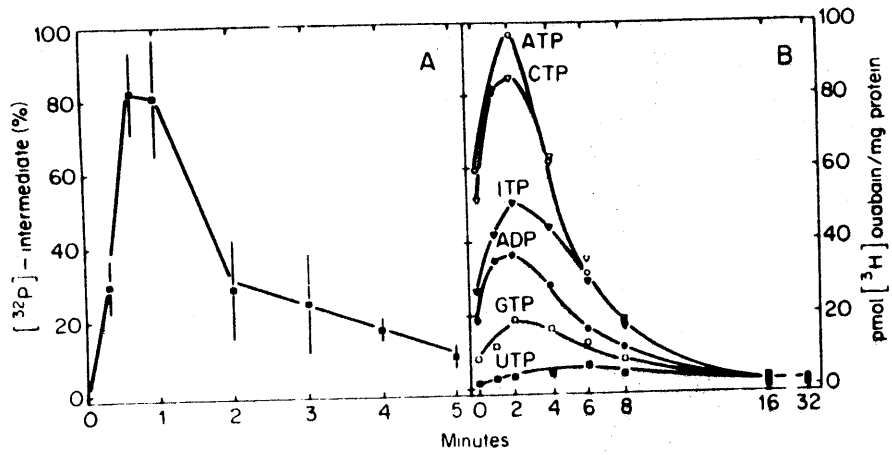


Fig 9 Alternate Hypothesis to Explain the Mechanism of ATP Stimulated (3H) Ouabain Binding

1. $E + ATP \longrightarrow E.ATP + OU \longrightarrow OU.E.A.T.P.$

2. $E + ATP \longrightarrow E\text{-}P + ADP + OU \longrightarrow OU.E\text{-}P$

In mechanism (1), simple binding of ATP to the enzyme stimulates ouabain binding, while in mechanism (2), Phosphorylation of the enzyme is necessary to stimulate ouabain binding.

Fig 10 Inhibition of the equilibrium level of {^{3}H} ouabain binding by β,γ-methylene ATP. Guinea-pig kidney enzymes were incubated with 5×10^{-7} M {^{3}H} ouabain at 37°. The binding reaction was started by the addition of 4 mM Mg^{++} at zero time and stopped at the indicated times. The solid circles (●) show binding in the presence of 4 mM Mg^{++}, 1 mM Pi, and 60 mM Na^+. After fifteen minutes, binding in the presence of Na^+, Mg^{++} and Pi was challenged by the addition of 3 mM ATP solid squares (■) or 3 mM β,γ-methylene ATP, open squares (□). The solid triangles (▲) show {^{3}H} ouabain binding in the presence of 60 mM Na^+, 3 mM Mg^{++} and 5 mM ATP and the inset triangle (see arrow) shows binding in the presence of 4 mM Mg^{++}, 1 mM Pi and 2.5×10^{-4} M unlabeled ouabain. Binding in the presence of Na^+, Mg^{++} and Pi at 30 minutes was arbitrarily taken as 100% Maximal binding 4 mM Mg^{++}, 1 mM Pi without Na^+ averaged 94.2 pmol {^{3}H} ouabain/mg protein. (Reprinted by permission from MOLECULAR PHARMACOLOGY 1973. 8:256.)

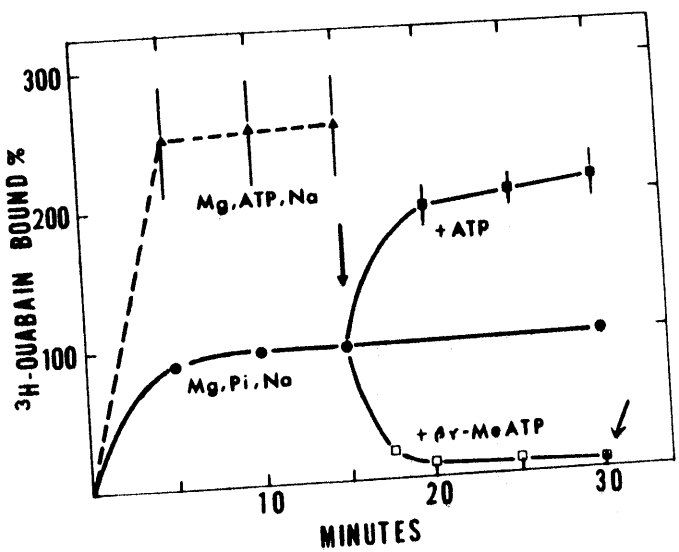


Fig 11 Stability of enzyme-ouabain complex at different temperatures. Enzyme, 4 mM $MgCl_2$, 1 mM H_3PO_4 and 2.5 x 10^{-7} M {^3H} ouabain were incubated for 20 min at 37°. The Tubes were then cooled to the indicated temperature and 10 mM EDTA and 5 x 10^{-4} M unlabeled ouabain added. The reaction was stopped by centrifugation at the indicated time points. At 22 and 37° only unlabeled ouabain was added. At 37° the reaction was stopped by rapidly freezing (20 sec) the tubes in an acetone-dry ice mixture. After thawing at 0° the tubes were centrifuged as before. The pH of the system did not change (7.4 ± 0.1 pH units) with temperature. (Reprinted by permission from Tobin and Sen, 1970).

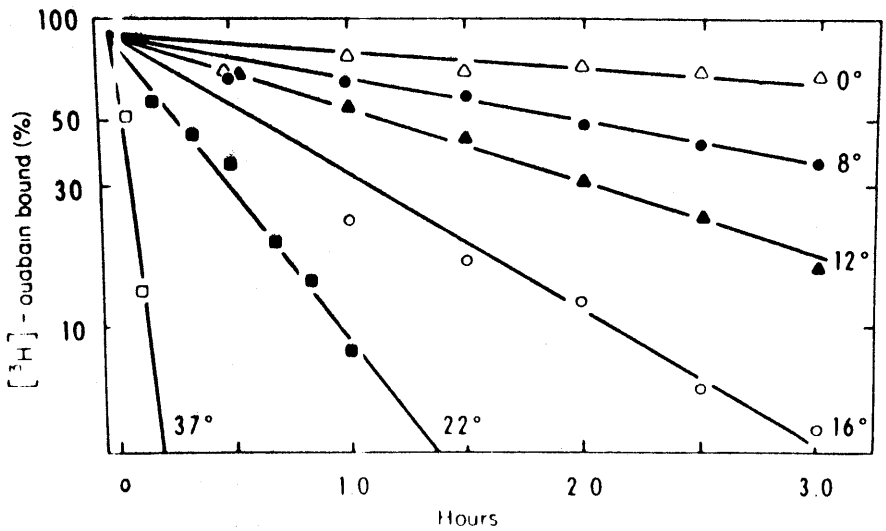


Fig 12

Dissociation of enzyme-ouabain complex in different tissues and species: [^{3}H] ouabain-enzyme was formed and its dissociation followed as described by Tobin et al. (1972b). The symbols indicate the percentage of the binding at zero time in a given tissue remaining at the times indicated. The enzymes prepared from different tissues were as follows: ▲, guinea-pig kidney; ●, guinea-pig heart; ⊡, guinea-pig brain; ■, dog heart; ♦, dog kidney; 0, cat heart.
Reprinted by permission from Tobin et al (1972b).

Thomas Tobin and Tai Akera

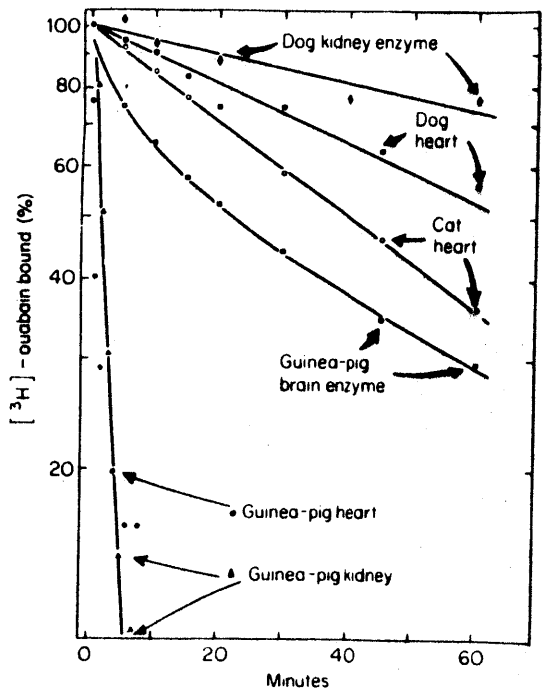


Fig 13 Dissociation of [^{3}H]-ouabain from cardiac Na^++K^+-ATPase *in vitro* at 27° C. Bound [^{3}H]-ouabain was expressed as the percentage of that immediately after the addition of unlabeled ouabain in each enzyme preparation. ●, dog; O, cat; ▲, rabbit; and ■ guinea-pig. Each point represents the mean of five experiments. Vertical lines indicate SEM.

(Reprinted by permission from Akera *et al*, 1973).

Fig 13 Dissociation of {^{3}H}-ouabain from cardiac Na^+ +K^+-ATPase in vitro at 27° C. Bound {^{3}H}-ouabain was expressed as the percentage of that immediately after the addition of unlabeled ouabain in each enzyme preparation. ●, dog; O, cat; ▲, rabbit; and ■ guinea-pig. Each point represents the mean of five experiments. Vertical lines indicate SEM. (Reprinted by permission from Akera et al, 1973)

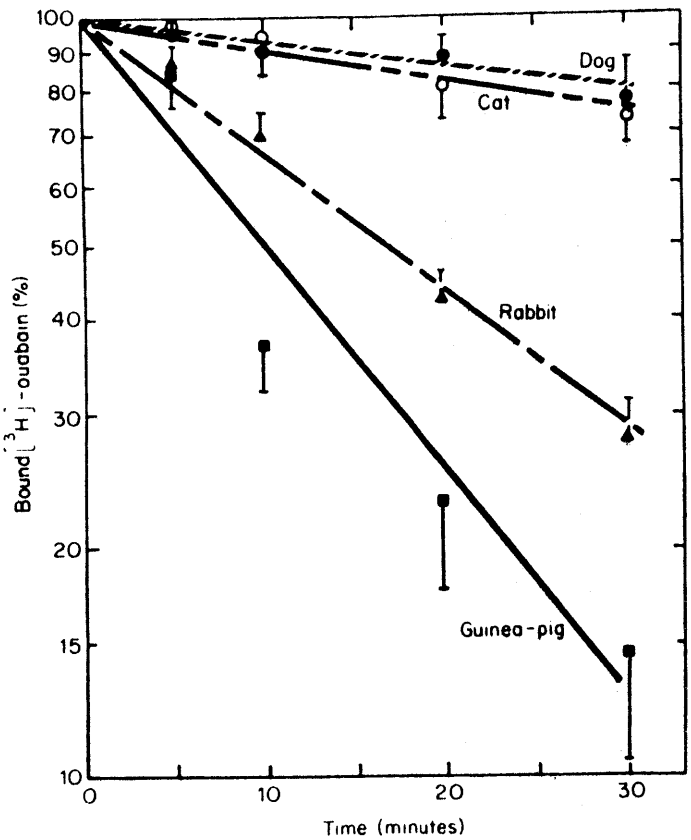


Fig 15 Half-times of the inotropic effect of ouabain and the dissociation of [^{3}H]-ouabain from cardiac ($Na^+ + K^+$)-activated ATPase at 27° C. Half-time was calculated from Fig 12.10 and 12.11 by fitting a linear regression line to the data obtained in each experiment. Mean of four (inotropic data) and five (dissociation data) experiments in each species, respectively. Vertical lines indicate SEM. *Reprinted by permission from Akera et al (1973).*

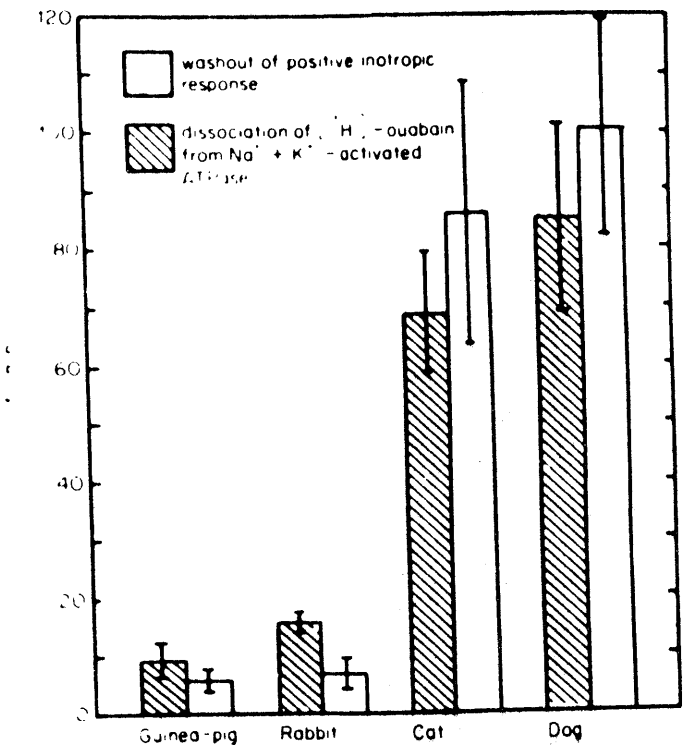


Fig 14 Washout of positive inotropic effect of ouabain in Langendorff preparations at 27° C. The maximal positive inotropic effect was arbitrarily set at 100 per cent. ● dogs; 0 cats; ▲ rabbits; and ■ guinea-pigs Each point represents the mean of four experiments. Vertical lines indicate SEM. *Reprinted by permission from Akera et al (1973).*

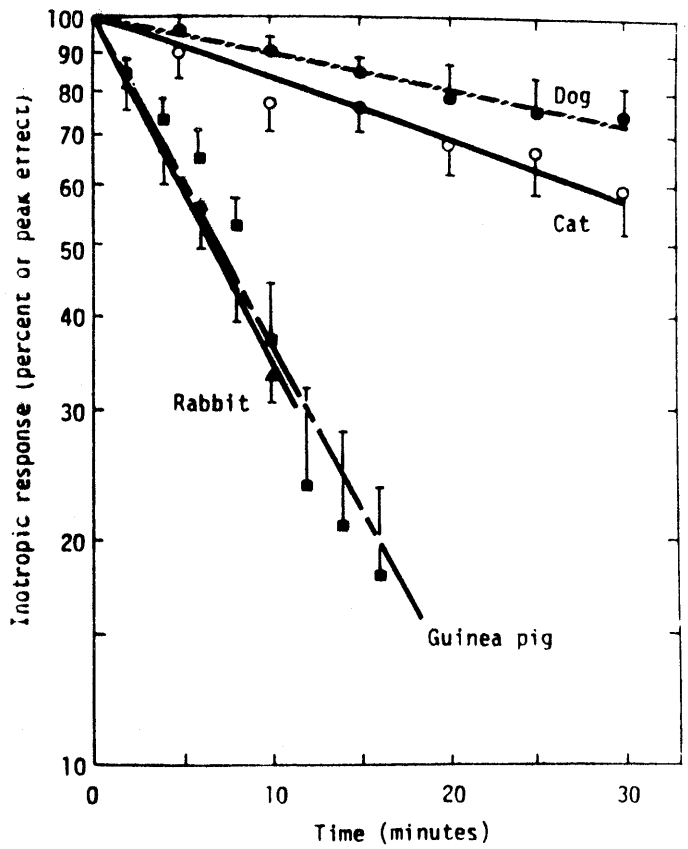


Fig 16 The onset and duration of the positive inotropic action of ouabain and digitoxin in electrically-driven isolated guinea-pig hearts. After (a) 45 min equilibration period, either 1.2 μM ouabain or 0.4 μM digitoxin was perfused for 10 (b) or 20 (c) min; 20-min drug-perfusion was followed by perfusion with drug-free solution for 8 (d), 24 (e) or 40 (f) min, respecitvely. Each point represents the mean of at least 5 experiments. Vertical lines indicate standard error of the mean. Arrows indicate the time points at which perfusion was terminated for the experiments shown in the subsequent figures. (Reproduced with permission from Ku et al, 1974)

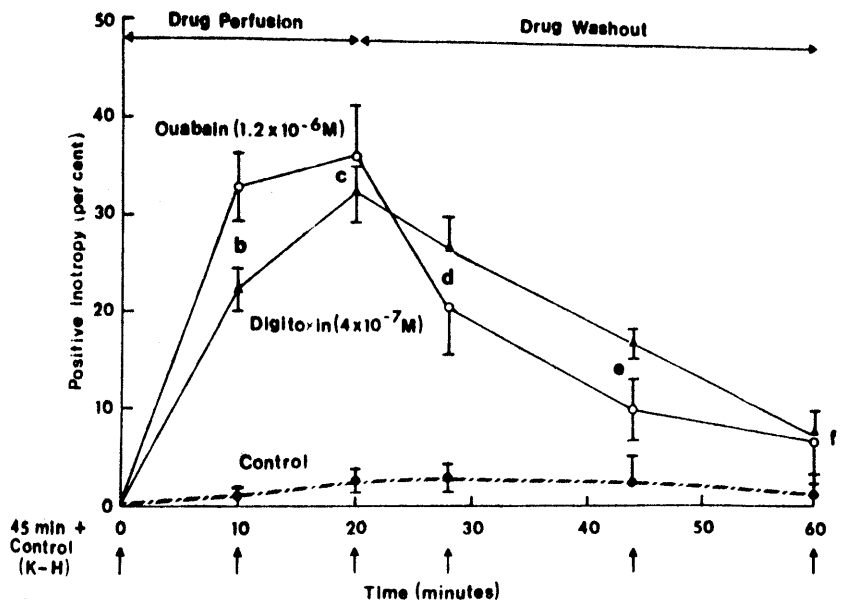


Fig 17 Correlation between the initial velocity of specific [^{3}H]-ouabain binding and increases in cardiac contractile force in digitoxin-perfused guinea-pig hearts. See the legend to Fig 16. The isolated hearts were perfused with digitoxin instead of ouabain. Each point represents the mean of at least 5 experiments. The control value represents the mean of 30 experiments, 5 to 6 experiments for each corresponding drug-perfused and drug-washout time point. *Reproduced from Ku et al (1974).*

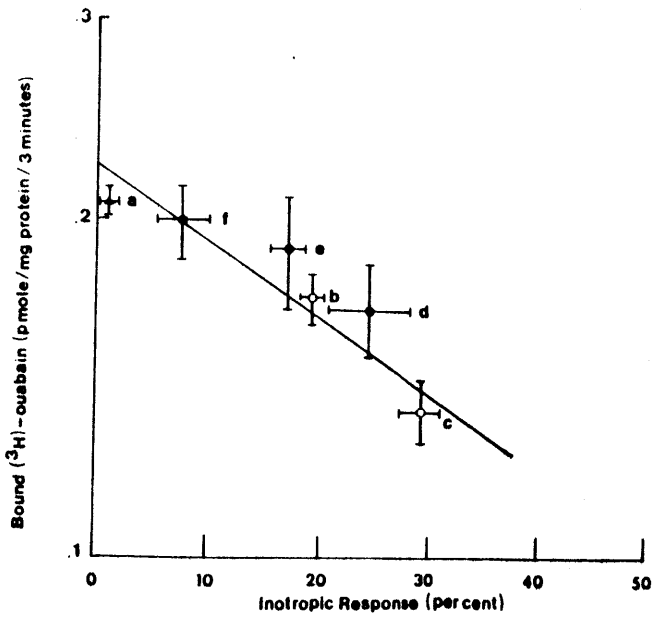


Fig 18 Correlation between the monovalent cation pump activity and changes in cardiac contractile force in digitoxin-perfused guinea-pig hearts. The cation pump activity was assayed from ouabain-sensitive ^{86}Rb uptake by the ventricular slices. After a 5-min preincubation of medium at 37° C for temperature equilibrium, the reaction was started by the addition of two ventricular slices (approximately 25 to 50 mg of tissue per slice) which had been previously incubated at 0° C in K+-free solution. Amount of ^{86}Rb uptake in the presence of ouabain after 8 min were subtracted from that obtained in the absence of ouabain to calculate the ouabain-sensitive ^{86}Rb uptake. The regression line for 32 points on semi-logarithmic plot was calculated by the least squares method. The closed triangle (point *a*) represents the mean value of 25 control hearts, 5 experiments for each corresponding drug-perfused and drug-washout time points. Points *b* and *c* represent data obtained from hearts perfused with digitoxin for 10 and 20 min, respectively. Points *d*, *e* and *f* are values after 20 min digitoxin perfusion followed by washout of 8, 24, and 40 min, respectively. Vertical lines and horizontal lines indicate standard errors of the mean of the changes in ^{86}Rb uptake and changes in cardiac contractile force, respectively. Each point represents the mean of 5 experiments.

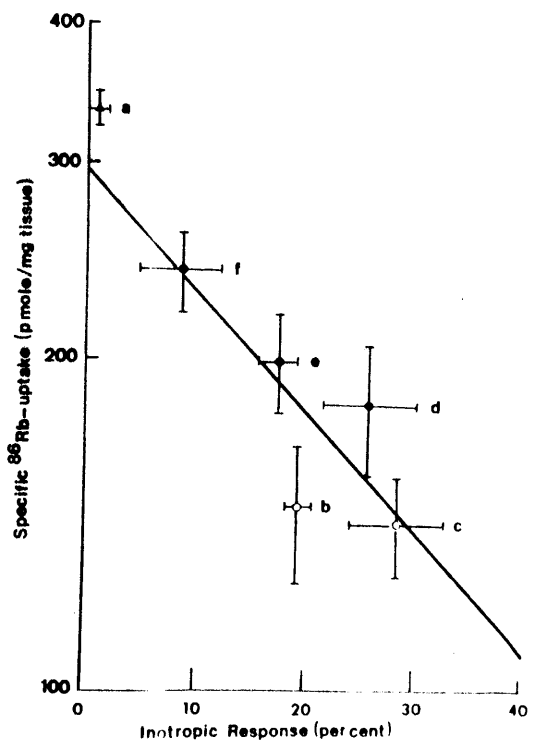


Fig 19 Photochemical reactivity of 3-azidoacetyl-strophanthidin.

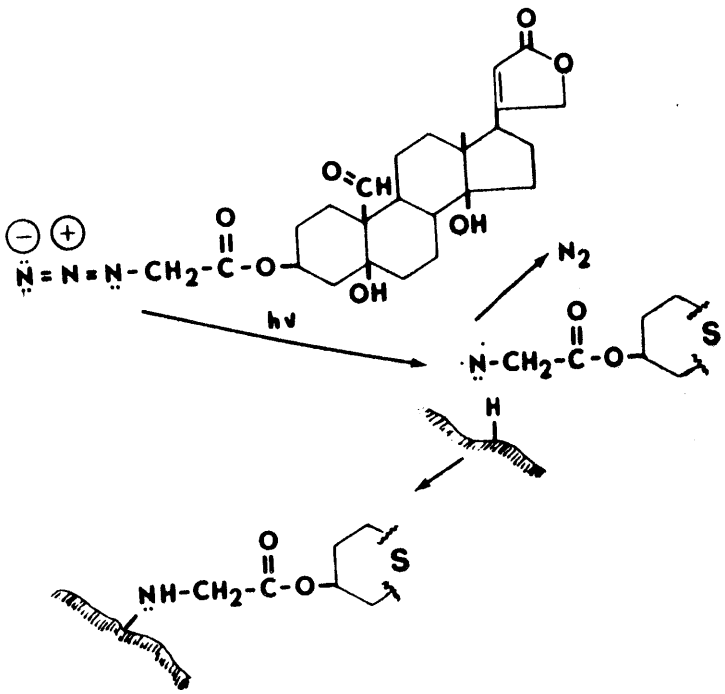


Fig 20 Inhibition of $Na^+ + K^+$-ATPase by 3-azidoacetylstrophanthidin and strophanthidin. About 40 µg of rat brain $Na^+ + K^+$-ATPase preparation were incubated with 100mM Na^+, 5 mM Mg^{++}-ATP and 15 mM K^+ in the presence of the indicated concentration of strophanthidin (O-O) or 3-azidoacetyl strophanthidin (●-●). $Na^+ + K^+$-ATPase activity is expressed as a percentage of that observed in the absence of added drugs, which averaged 296 + 3 µM Pi/mg protein/hr. All points are single experimental determinations except those at 10^{-6} M drug, which are the means ± SEM of determinations on 4 different enzyme preparations.

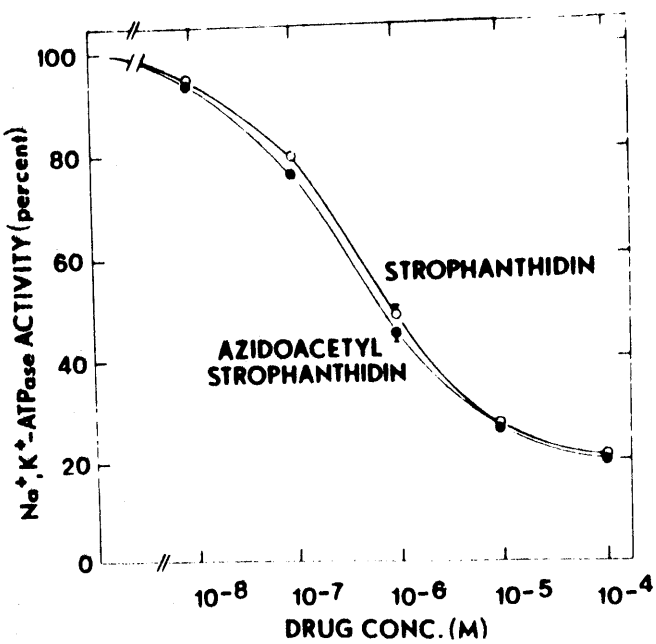


Fig 21 Cardiotonic activity of AAS on isolated electrically driven guinea pig atria. The upper panel shows typical responses of guinea pig atria to the indicated concentration of AAS in the bathing medium. The lower panel shows the responses of five different guinea pig atria to the indicated concentration of AAS in the bathing medium. In each case the bathing medium was Krebs-Henseleit solution (145 mM Na^+, 58 mM K^+) at 30° C and the atria were electrically driven at 1 Hz.

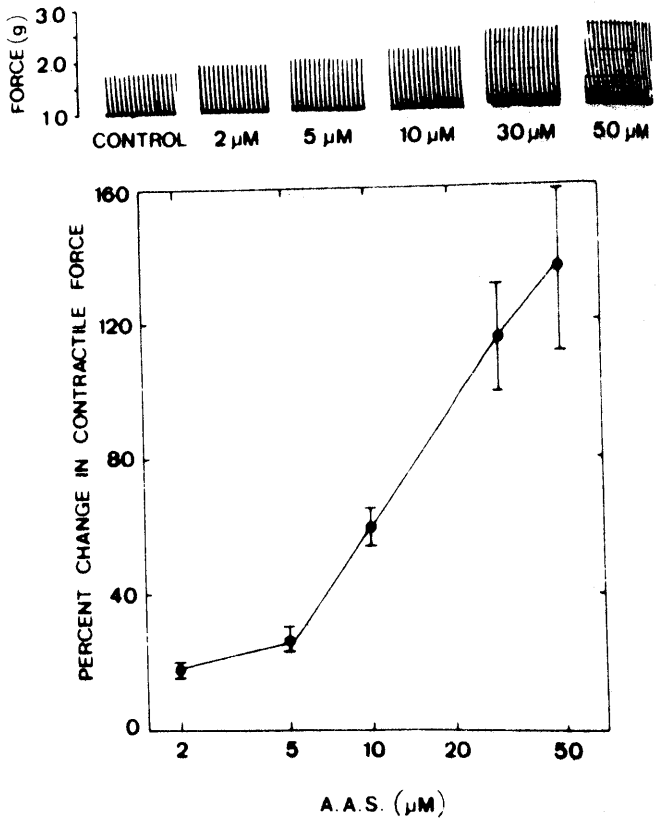


Fig 22 Effect of fentanyl on spontaneous motor activity in a single horse. A horse was injected with saline or fentanyl IV. The number of times the horse took a step with its left front foot in a 2-min period was recorded as an indication of locomotor activity. Activity following saline injection is shown in the bottom panel by the solid circles (●-●) and activity following fentanyl doses ranging between 0.001 and 0.040 mg/kg is diagrammed in the upper panel.

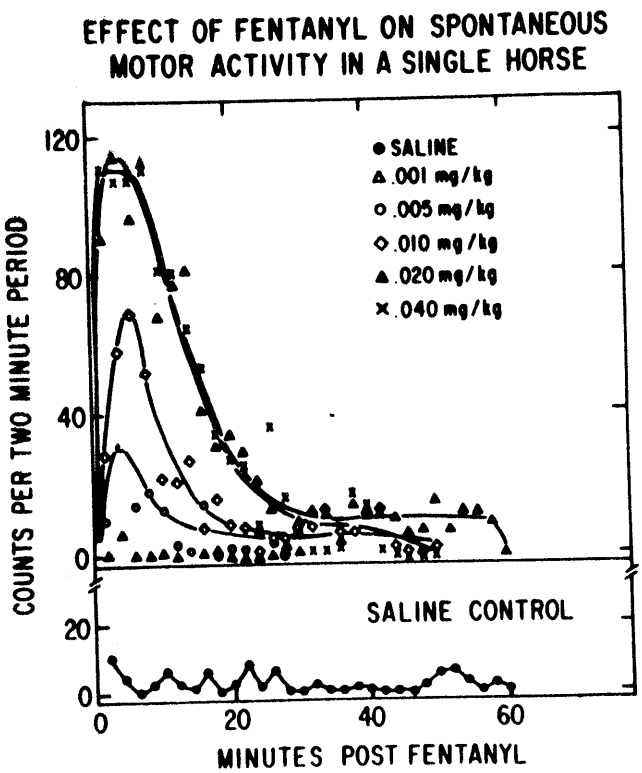


Fig 23 Effect of etorphine on spontaneous locomotor activity of four horses. Four horses were injected IV with saline and then increasing doses of etorphine. The locomotor response to 0.00002 mg/kg etorphine is shown by the open circles (0-0); to 0.00010 mg/kg, by open squares (□-□); to 0.00020 mg/kg by closed circles (●-●); and to 0.00040 mg/kg by crosses (x-x). The straight line at 4 counts/2-min period represents the average response to saline.

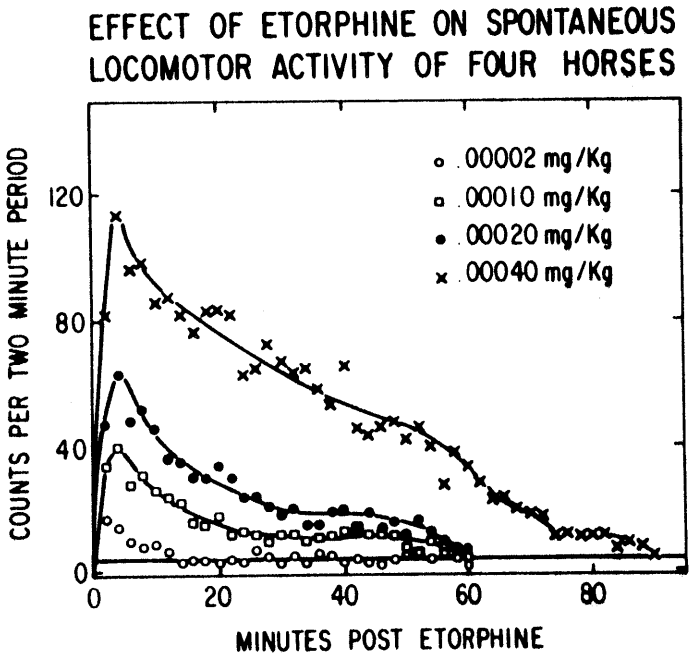


Fig 24 Effect of meperidine on spontaneous motor activity in the horse.
A single horse was injected IV with saline and three different dosage levels of meperidine. The average counts per 2 mins following dosing with saline are shown by the straight line near the bottom of the graph. After injection of the drug, average counts per 2-min period were determined for the 16-min interval immediately preceding the time indicated.

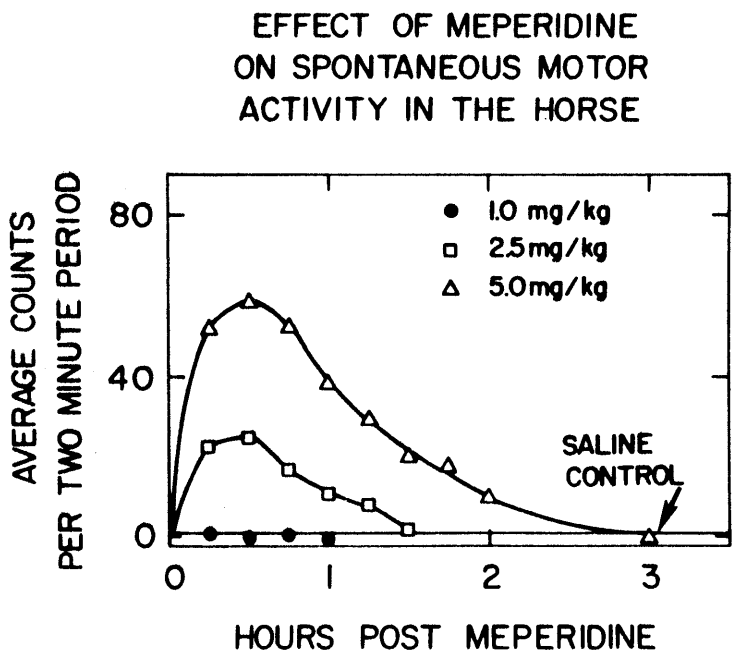
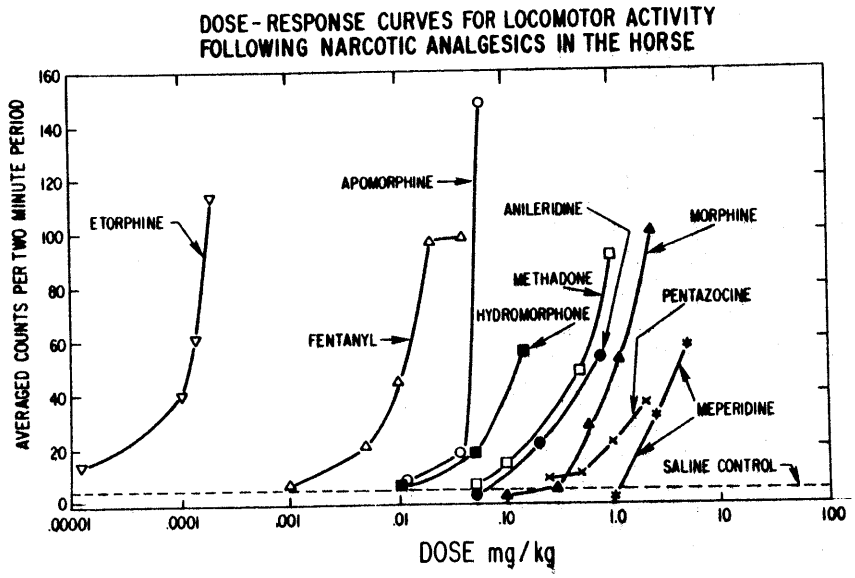


Fig 25 Dose response curves for locomotor activity following narcotic analgesics in the horse.

Horses were dosed with increasing amounts of the indicated drugs and the average number of steps taken during the peak 2-min period were plotted for etorphine, fentanyl and apomorphine. For all other drugs, average counts per 2-min period were determined for the 16 min interval of peak activity. Three to ten horses were used in the experiments on etorphine, fentanyl, apomorphine, methadone, morphine and pentazocine. One horse was used to determine each dose response curve for hydromorphone, anileridine and meperidine. The average counts for 2-min period for the saline control are shown by the dashed line near the bottom of the graph.



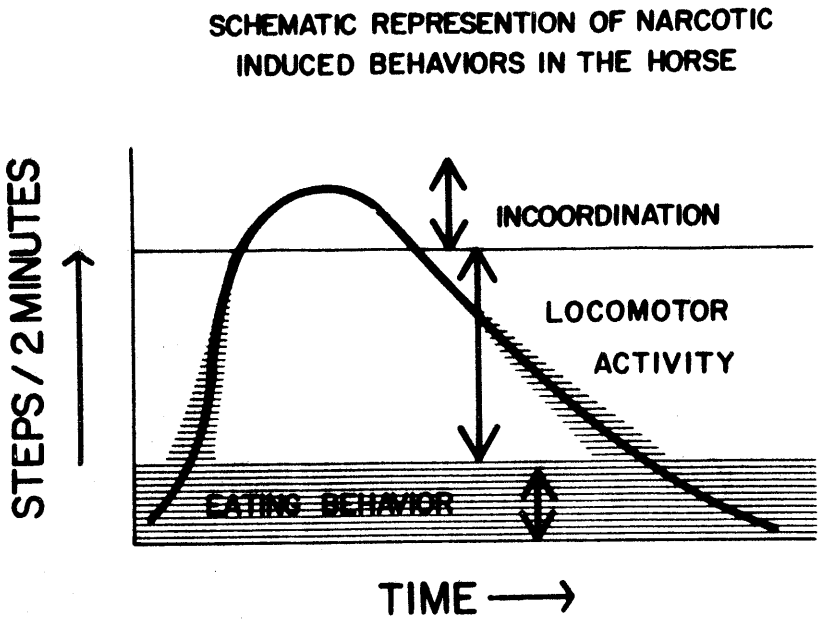


Fig 26 Schematic Representation of Narcotic Induced Behaviors in the Horse.

The curved line represents a typical locomotor activity response by a horse to a large dose of a narcotic drug. At lower dose rates, or at the beginning or end of a response, eating behavior predominates, indicated by the shaded area. As the dose of drug, and presumably its plasma levels, increase, locomotor activity becomes predominant. At very high doses of drug, incoordination limits the locomotor response.

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