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THE DISTRIBUTION AND RELEASE OF CALCIUM IN FROG NERVE

by

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INTRODUCTION

It has long been known that nerve axons exhibit spontaneous repetitive discharge in low-calcium or calcium-free media (Arvanitaki, 1939; Katz 1936; Brink et al.,1946). In 1901 Loeb described a new type of irritability caused by the addition of calcium precipitants to either the nerve or muscle portion of a nerve-muscle preparation: "the muscle twitched rythmically and then shortened steadily until tetanus occurred" (Loeb, 1901). In the following years work was done to examine the action of calcium deficiency on the electrical phenomena of excitable tissue. Experiments on squid axons (Arvanitaki, 1939), crab nerves (Katz, 1936) and small bundles or single fibers of desheathed frog nerves (Brink et al., 1946; Brink and Bronk, 1941) showed that low calcium gives rise to fluctuating circulating currents, recorded as cyclic local variations of membrane potential. This could be observed by stimulating with subthreshold current in a low-calcium solution or simply by further removal of calcium. The amplitude of the local discharge was inversely related to the concentration of calcium in the medium, and in time spontaneous propagated impulses

could be obtained, the frequency being related to the frequency of the local oscillatory variations. The initial effect of calcium deficiency is then the appearance of oscillatory activity. Multiple spikes arise from oscillatory subthreshold responses when they reach sufficient magnitude and rate of rise.

Significance of Spontaneous Discharge in Calcium-Free Solutions

The observed spontaneous discharge indicates that membrane calcium controls the transmembrane movement of current-carrying ions like sodium and potassium. Evidence for a membrane-bound calcium comes from various sources. Since the days of Ringer it has been known that external calcium is necessary for normal excitability (Ringer, 1882; Ringer, 1885). The work of Heilbrunn (1943) suggested that calcium is found on the surface of all cells, and he stressed its importance in various cell functions. Calcium is needed for surface precipitation reactions (Heilbrunn, 1956), for adhesion between cells (Ringer, 1890) and for maintenance of their overall integrity (McCutcheon and Lucke, 1928; Bolingbroke and Maizels, 1959; Manery, 1966). Thus, if a cell is pricked, cytoplasm will flow out in the absence of

calcium; cells tend to separate in such a medium; and the permeability to water, potassium and sodium and even larger molecules such as proteins increases in various tissues bathed in calcium-free solutions. After addition of calcium these effects will reverse if the pH is appropriate for binding of the ion (Steinberg, 1958). Microincineration studies of myelinated frog nerve with subsequent microscopy showed calcium to be found at the periphery and perhaps membrane of the axons (Scott, 1940). The possibility arises, therefore, that membrane calcium is intimately related to processes of excitation, as evidenced by the occurrence of spontaneous activity after calcium removal.

Calcium is probably bound to fixed negative charges on protein or lipoprotein of the membrane (Mazia, 1940). It is known that calcium is bound by proteins (Klotz, 1946), lipids (Drinker and Zinsser, 1943) artificial membranes composed of lipids (Kimizuka and Koketsu, 1962) and by isolated cell membranes (Koketsu et al., 1964). The two charges of the ion could be bound by a single molecule, in which case calcium could control molecular conformation (Rice, 1959) and/or charge density of the surface (Teorell, 1949, or calcium may link adjacent acidic macromolecules

and function as a gating mechanism (Gordon and Welsh, 1948). Thus, one calcium ion could bind to the oxygens of two radially oriented phospholipids and bridge a membrane channel. Another possibility which has been considered is the binding of a Ca-ATP complex to a membrane macromolecule (Abood et al., 1964). Coordination complexes binding many calcium ions and many ATP molecules to a protein or lipoprotein could also occur (Abood and Gabel, 1965). Abood considers such complexation best suited for the reversible chemical processes associated with axon activity.

Compartmentalization of Cell Calcium

Total tissue calcium studies in frog nerve revealed two components of calcium: one comprises 40% of the total and is exchangeable; a second is a tightly bound non-exchangeable fraction, comprising 60% of the total (Tipton, 1934). Efflux studies with radioactive calcium from crab nerve showed the exchangeable fraction to be composed of two components, a rapidly effluxing one labelled as extracellular in origin and a slower one thought to be derived from a cellular source (Soloway et al., 1953). Work with radiocalcium and measurements of calcium content in muscle also showed these three components (Gilbert and Fenn, 1957; Grossman and Furchgott,

1964) - an extracellular component, a cellular component exchangeable with extracellular cations and the non-exchangeable fraction. An additional component that is exchangeable only with calcium of the medium (self-exchangeable calcium) and thought to be surface bound, has also been revealed in muscle (Shane and Bianchi, 1959).

Role of Calcium in Excitation

In addition to spontaneous activity, decreased external calcium brings about a lowering of excitation threshold (Brink et al., 1946), slower accommodation (Solandt, 1936), a decrease in the amplitude of the action potential (Frankenhäuser, 1957) and decreased rectification (Steinbach et al., 1944). Calcium removal causes depolarization in some tissues such as isolated frog nerve axons (Staempfli and Nishie, 1956), while in other preparations this cannot be demonstrated (Koketsu and Koyama, 1962). Removal of all external calcium causes inexcitability of isolated axons (Frankenhäuser, 1957). Important contributions to the understanding of the function of calcium in excitation were (1) the finding by Cole (1949) that the resistance of squid axons varies directly with the concentration of external

calcium, and (2) the work of Frankenhäuser and Hodgkin (1957) showing that membrane depolarization and calcium removal are roughly equivalent. Thus, low external calcium promotes sodium entry so that a smaller depolarization is required to produce a given rise in sodium conductance; raises the rate at which sodium current increases at the beginning of depolarization; increases the maximum sodium current. Similarly, low-calcium increases potassium permeability, increases the rate of rise of potassium conductance and hastens its onset. Frankenhäuser and Hodgkin also found that sodium inactivation was increased in low-calcium solution.

Because calcium was found to have a role in depolarization, and depolarization could be thought of as producing the removal of calcium from some site on the membrane (Gordon and Welsh, 1948), more direct evidence was sought for this contention with the use of tissues labelled with radiocalcium and subjected to excitable stimuli. Hodgkin and Keynes (1957) showed that an increased influx of radiocalcium was produced by electrical stimulation of squid axons. This was confirmed in muscle in which 30 times more calcium flows in per stimulus than in nerve (Bianchi and Shanes, 1958). Increased efflux of calcium was shown to occur in desheathed frog nerve and

spinal roots by Koketsu and Miyamato (1951). Increased external potassium, another depolarizing stimulus, also produces an increased efflux of calcium (an Breeman and Daniel, 1966). This pointed to a simple displacement of calcium by potassium. That the calcium moving at the time of stimulation comes from the surface of the membrane was deduced from the fact that no increased efflux is seen when radiocalcium is injected into the cell before stimulation (Hodgkin and Keynes, 1957), and that radiocalcium must be administered some minutes before potassium in order to obtain any effect (Bianchi and Shanes, 1959).

Tobias (1964) proposed a molecular basis for excitation that takes into account the movement of calcium during excitation and its displacement by potassium: "the early catelectrotonic outward exciting current moves potassium from the axoplasm into the membrane phase displacing calcium"; this causes changes in the membrane that lead to increased permeability to sodium and potassium. The basis for such a calcium displacement also evolved from studies of membrane constituents and their interaction with ions. Monolayers of lecithin bind calcium at physiological pH in a constant binding ratio; the addition of KCl and NaCl reduced this

binding (Palmer and Schmitt, 1941). In the presence of potassium and other monovalent cations, lipid emulsions have a reduced water content but calcium removes water almost completely (Palmer and Schmitt, 1941). This seemed to be a clue of calcium action and has been studied further. After adding cholesterol to phospholipid mixtures, the water content and electrical resistance could be manipulated by the addition of ions (Leitch and Tobias, 1964). Calcium was taken up preferentially in the presence of sodium and potassium. The injection of potassium into a calcium membrane model by polarization increased permeability by increasing water content. The ability of the divalent ion to crosslink and therefore shrink the mass of the lipid mixture probably accounts for this effect. Phosphatidylserine was subsequently found to be the lipid responsible for the interaction with the ions (Nash and Tobias, 1964). The importance of the model work was revealed when red blood cell ghost membranes and microsomes were shown to have similar properties: calcium is taken up preferentially in the presence of the other ions; it decreases water content; and the predominant ion in the membrane determines

membrane resistance (Carvalho et al., 1963). The first step in excitation according to Tobias (1964) is then a displacement of calcium which loosens the membrane by increased water content.

In addition to the spreading apart of the lipid portion of the membrane due to potassium infiltration, Tobias also proposed a change in geometry of the proteins at the time of stimulation. That proteins are involved in the excitation phenomenon was suspected by Rojas (1965) who showed that internal injection of proteolytic enzymes into squid axons abolished the action potential. Inexcitability can also be produced by treatment with sulfhydryl inhibitors, which also suggests a requirement for structural integrity of proteins (Smith, 1958; Huneeus-Cox et al., 1966). A recent finding that antibodies against squid axoplasm proteins abolish excitability, adds to the evidence that supports a role of protein in excitation (Huneeus and Fernandez, 1967). The extraction of an actomyosin-like protein from various membranes (Onishi, 1962; Duncan, 1967), the action of ATP as a membrane stabilizer (Okamoto et al., 1964; Kuperman et al.,1967) and the recent work showing calcium uptake by crustacean nerve

subcellular membranes in the presence of ATP (Lieberman et al., 1967), point to the possibility that a configurational change involving membrane protein, ATP and calcium - similar to muscle contraction - may play a role in excitation.

In line with the importance of calcium in excitation and experiments showing that sodium is not required for excitability in perfused squid axons (Tasaki and Singer, 1966) Tasaki has proposed a theory for nerve activity somewhat different from the ionic theory of Hodgkin and Huxley, in which the dissociation and association of calcium is responsible for regulating the membrane potential: the membrane can maintain two physico-chemical stable states, a resting state where calcium is fully associated and an active state where calcium is dissociated from its binding sites (Tasaki, 1959). The action potential is generated during the transition between the two states and is determined by the rate and amount of dissociation and association of bound calcium.

The role of calcium as a current-carrying ion is a new aspect of the function of this ion in excitation. Crustacean and arthropod muscle produce feeble, non-propagated and graded

responses in normal sodium containing solutions, while prolonged action potentials are obtained with quaternary ammonium salts in sodium-free solutions containing calcium and magnesium (Werman and Grundfest, 1961). The organic cation is thought to produce an increased permeability for the alkali earth cations so that they carry the current. Some preparations have been found in which the rising phase of the action potential is directly proportional to external calcium concentration: the giant nerve cell of a snail and the muscle fibre of the barnacle show an overshoot in sodium-free solution that is directly related to calcium concentration in the medium (Hagiwara et al., 1964; Meves, 1965). Voltage clamp measurements revealed a rapid inward surge of calcium. The spike-generating mechanism in guinea pig Taenia coli was found to be the influx of calcium (Nonomura et al., 1966). Thus, calcium can, under certain conditions and in certain excitable cells, be a current carrying ion like sodium.

Interaction between Calcium and Pharmacological Agents

Studies of the interaction of calcium with pharmacological agents (including metabolic inhibitors) that affect

excitability provide further insight into the role of calcium in excitation. The interaction between oxidative metabolism and excitation has been explored in various ways for a long time. Lowered calcium in the medium produces an increased uptake of oxygen by nerve tissue (Brink, 1957) even before spontaneous activity begins. Abood (1954) showed that this is not due to enzyme activation and he proposed a complex set of phenomena involving changes in configuration of a membrane molecule (Abood, 1966). Van der Kloot (1967) has shown that the increased respiration due to potassium depolarization in frog sartorius muscle can best be related in time and magnitude to the inflow of calcium into the sarcoplasm. In any case, an immediate relation between excitability and metabolism has not been established (Abood et al., 1964). For example, the metabolic inhibitor 2, 4-dinitrophenol was found to have little effect on resting potential of squid axon or frog nerve fibres (Hodgkin and Keynes, 1955). Yet it depolarizes frog skeletal muscle rapidly (Ling and Gerard, 1949). Koketsu (1965) found that this agent releases calcium from skeletal muscle and he relates the depolarization to this effect.

Isolated sartorius muscle of frog is strongly depolarized in calcium-free solutions containing EDTA (Koketsu and Noda, 1962). An increased rapid transient efflux of calcium is also produced by this agent. This calcium was thought to be derived from a superficial component because its rate of efflux is the same as that of the initial fast component of calcium (Bianchi, 1961; Bianchi, 1965). Rectus abdominis muscle, however, gives a sustained slow increase of calcium efflux by the same agent and an accompanying sustained contracture not seen with sartorius muscle (Bianchi, 1965). The interpretation here was that the removal of a superficial calcium component brings about calcium movement from a deeper cellular site.

The interaction of caffeine with calcium has aroused interest because the addition of this agent to isolated muscle leads to contraction without changing the resting potential (Bianchi, 1963). Changes in the concentration of external calcium were ineffective in altering the response of muscle to caffeine (Bianchi, 1961), and so subsequent experiments were designed to test the action of caffeine on membrane calcium. Both influx and outflux of calcium were found to be increased by caffeine even after removal

of a fraction of calcium by EDTA (Bianchi, 1961). Further work showed that this agent interferes with binding sites and increases release of calcium in subcellular particles such as mitochondria and microsomes (Herz and Weber, 1965; Nayler and Hasker, 1966).

Berwick (1951) reported that ether, chloroform and cocaine release calcium from muscle tissue. Since cocaine is not a lipid solvent like the other two agents, she proposed that this was a specific anesthetic action. Using voltage clamp techniques, Shanes et al.,(1952) showed that procaine and high external calcium have common effects; both depress permeability to sodium and potassium during activity and increase threshold. However, procaine reduces spike height and rate of rise while calcium does not. Shanes interpreted this in the following manner: calcium occupies sites that become available to monovalent ions during activity; anesthetics either cause a reduction of such sites by interacting with them or penetrate between the channels of ion movement to exert restraint on configurational changes. Aceves and Machne (1963) were able to restore the spike by a 10-fold increase of external

calcium after the spike had been abolished by a local anesthetic in frog ganglion cells. The antagonistic action between the divalent cation and local anesthetics was also demonstrated in lobster axon (Goldman and Blaustein, 1966). Feinstein (1963) showed that procaine and other local anesthetics antagonize caffeine rigor and calcium self-exchange in frog skeletal muscle. Using phospholipid membrane models, Feinstein (1964) also showed that anesthetics inhibit calcium movement from an aqueous to a chloroform layer, increased resistance of model films and yielded a fall in pH when added to phospholipid sols; it was concluded that local anesthetics interfere with calcium binding sites.

PURPOSE

Knowledge about the roles of calcium in peripheral nerve function has come mainly from studying the effects of changes in the external calcium concentration on electrophysiological activity. Such studies have established that calcium deficient solutions cause membrane depolarization, inexcitability and most characteristically, spontaneous repetitive discharge. These observations suggest that calcium plays several different roles in the excitable cell: (1) controls membrane permeability, (2) controls sodium inactivation and (3) is released from the membrane by depolarizing stimuli. Although the importance of calcium in normal axonal function has been convincingly demonstrated, the specific mechanisms of calcium action, either at rest or during excitation and drug treatments, are still largely matters of speculation and hence open to further experimentation. An initial important step is to obtain data on calcium content and fluxes in axons that are subjected to various types of chemical and physical environments and correlate this data with simultaneous alterations in electrophysiological activity, especially spontaneous activity. This was the major aim of the experiments reported herein.

METHODS

Measurements of electrical activity, calcium fluxes and calcium content were carried out on sciatic-peroneal nerves isolated from the frog Rana pipiens at all seasons of the year. Shortly after arrival, the frogs were placed in tap water at 5°C , and they were maintained in this manner for 1 to 60 days prior to an experiment. Depending on the experiment, the nerves were used either with surrounding epineural sheath intact (ensheathed nerve) or the sheath was carefully removed (desheathed nerve). Such desheathing was usually done immediately after isolation of the nerve.

The normal frog Ringer solution into which all nerves were first immersed after isolation or desheathing contained (mM): 110.88 NaCl, 2.0 KCl, 1.8 $CaCl_2$, 0.1 NaH_2PO_4 and 2.02 $NaHCO_3$; pH 7.2; 22-24°C . Calcium-free Ringer's was prepared without the addition of $CaCl_2$. Deionized water (Continental Water Corp.) was used to prepare all solutions. As determined by atomic absorption spectroscopy, the concentration of calcium in the calcium-free Ringer's was less than 10^{-6}M.

All test compounds were dissolved in the normal or calcium-free Ringer's and, if necessary, the pH was readjusted to 7.2 by the use of HCl or crystalline Tris (tris (hydroxymethyl)-aminomethane). Test solutions were prepared freshly for each experiment.

Electrical recordings. Action potentials were recorded in air or in light mineral oil at room temperature (22-24°C) (unless otherwise indicated) using conventional electrophysiological techniques. Stimulating and recording electrodes were made of platinum-iridium wire. The spontaneous and evoked potentials were recorded diphasically, the distance between the two recording electrodes being 15 mm. The intensity of spontaneous discharge was measured by estimating the average amplitude of the impulses directly from the oscilloscope screen or from photographs thereof, or by summating 10 successive sweeps (5 milliseconds/centimeter) of spontaneous impulses on the screen of a storage oscilloscope (Tektronix type 564). This kind of measurement takes into account the number of axons discharging and the degree of temporal summation of discharge in the axonal population. Evoked potentials were initiated by rectangular pulses of 0.01 millisecond duration applied at a rate of 0.5 per second.

Stimulus voltage was just supramaximal for the alpha-A fiber group. Excitability threshold was approximated by determining the stimulus voltage which produced a 20 microvolt action potential at a pulse duration of 0.01 millisecond. This magnitude of action potential was the smallest which could be accurately measured under the present experimental conditions, and it probably represents the response of a single fiber. A more useful measure of excitability in a multifiber nerve preparation was adopted in later experiments, i.e. the stimulus voltage required to produce 50% of the maximal alpha-A spike height recorded in nerves in normal Ringer solution.

Measurements of calcium content. To determine the effects of variation in external calcium concentration on the calcium content of nerve, freshly isolated nerves were first immersed in normal Ringer's for 4 hours and then for various predetermined time periods in the test Ringer's (10 ml. volume for each nerve). Control nerves were immersed in the same volume of normal Ringer's for the same periods of time. To determine the effects of various drugs on calcium content, paired nerves were used, i.e. the nerve isolated

from one leg of the frog served as a control, and the nerve from the opposite leg was the test nerve. The freshly isolated pair was first placed in normal Ringer's for 4 hours; one of the pair was then soaked for a prescribed period of time in 10 ml. of the drug - containing solution while the other nerve was changed to a 10 ml. normal Ringer bath for the same period of time.

After soaking in the various normal and test solutions, each nerve was rinsed for several seconds in a large volume of calcium-free Ringer's and blotted gently on ashless filter paper. The ligated ends were cut off, and the remaining segment of nerve was weighed in a preweighed container within 60 seconds. For dry weights, the nerve was dried in a silica crucible (18 hours; 100°C) and then re-weighed.

In the early experiments on calcium determination, the EGTA colorimetric titration technique, using HHSNN as indicator, was used (Chrambach et al., 1961 as modified by Weber and Herz, 1963). For the later and majority of experiments, an atomic absorption spectrometer was available. Phosphate interference was eliminated by the use of lanthanum so that

ashed samples could be used. Ashing was carried out at 600° C for 12 hours; platinum or silica crucibles were used, and each crucible contained 3 pre-weighed nerves. After removal from the furnace and cooling, 0.2 ml. of concentrated HCl was added to each crucible, and about 2 hours (with occasional stirring) was allowed for the ash to be dissolved. Lanthanum was added to obtain a final concentration of 0.5%, and the final volume of 2 ml. was made up with deionized water. After mixing, the calcium of the sample was determined with the absorption spectrophotometer set for zero absorption in the hot mode. The reagent blank contained acid and lanthanum in the same concentrations as the ashed samples.

Along with each run of unknown samples, a standard calcium curve was obtained. Calcium standards were prepared as follows: $CaCO_3$ was dried overnight at 110° C , weighed out, placed in a volumetric flask containing a small volume of concentrated HCl and made up to final volume with deionized water.

All glassware and crucibles used in the experiments on calcium measurement were soaked in 3N HCl before rinsing with deionized water.

Measurement of Ca-45 efflux. Ca-45 was obtained from New England Nuclear and made up to a stock solution of 20 μC./ml. in calcium-free Ringer's; an amount of $CaCl_2$ was then added to bring the final concentration of calcium to 1.8 mM (normal Ringer's). In order to label the slow component of the Ca-45 efflux adequately, nerves were soaked overnight at 5°C in 10 ml. of the 20 μC./ml. solution. After removal from this solution, each nerve was rapidly rinsed in 25 ml. of non-radioactive Ringer's. If the washout experiment was to be done on a desheathed nerve or isolated sheath, the desheathing was accomplished immediately after the rinse in non-radioactive Ringer's; then the washout study of the whole nerve, desheathed nerve or isolated sheath was carried out as follows: The tissue was placed in a chamber containing 2 ml. of calcium-free or normal Ringer's, depending on the experiment. This collection fluid was changed for fresh solution at 10 minute intervals throughout the entire washout period. Each 10 minute collection was emptied into a polyethylene scintillation vial, and 20 ml. of scintillation fluor was added. At certain times during the slow component of Ca-45 efflux (to be indicated for each experiment), the normal or calcium-free collection fluid

could be changed for one which contained the desired test agent. At the end of the entire washout period (which varied with the particular experiment), the tissue was ashed at 600°C , dissolved in 0.1 N HCl, and a 2 ml. sample was mixed with 20 ml. of scintillation fluor. Each 10 minute collection and the acid-dissolved ash obtained at the end of the washout period was counted in a liquid scintillation spectrometer, efficiencies and quench corrections being determined by the channels ratio method. All counts were corrected for radioactive decay, and the background count was subtracted automatically.

The scintillation "cocktail" was made up as follows: in 6 liters of p-dioxane (spectroquality) were dissolved 180g. naphthalene, 36g. 2,5 - diphenyloxazole, 1.8g.1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, and 600 ml. 2-ethoxyethanol.

From the data obtained in the Ca-45 washout experiments, the following types of curves were plotted: (1) a rate curve which describes the time course of the rate of efflux; (2) a desaturation curve which describes the decline of tissue

radioactivity, expressed as a percentage of the initial total radioactivity as a function of time; (3) a rate coefficient curve which is the percentage of the average radioactivity in the tissue during the time of collection that has emerged per minute during the collection period; (4) a relative efflux which is derived by dividing the radioactivity washed out during any 10 minute collection period by that which is washed out during a 20 minute collection period chosen as the reference base.

Determinations of extracellular space. C-14 labeled sucrose and inulin (New England Nuclear) were used for space determinations. Nerves were soaked overnight at 5°C in the radioactive solutions, rinsed with 25 ml. non-radioactive Ringer's, then gently blotted and weighed. Each nerve was then placed for 12 hours in 1 ml. deonized water in order to leach out the radioactive material. The nerve was homogenized in 2 ml. deonized water, centrifuged, and the supernatant was combined with the leaching fluid. One ml. aliquots of these solutions were mixed with 10 ml. of the scintillation fluor and counted in the liquid scintillation spectrometer. To insure that all the radioactivity had been removed by the

aforementioned procedures, 0.5 ml. NaOH was added to the centrifuged pellet, and this was heated to 100°C. This mixture was then centrifuged and the supernatant counted. With C-14 labeled inulin, this supernatant count was negligible while with C-14 labeled sucrose an additional count of about 6% was obtained. The space calculation was as follows:

$$\frac{\text{counts per minute/grams wet weight}}{\text{counts per minute/ml. radioactive solution}} = \text{ml./gram wet weight}$$

The washout of C-14 labeled sucrose and inulin into normal or calcium-free Ringer's was also studied in the manner described above for Ca-45. This served as a control for the Ca-45 efflux experiments and also provided data concerning the complexity of nerve extracellular space.

MATERIALS

(1) Organic compounds

caffeine sodium benzoate

2, 4-dinitrophenol (DNP)

dibucaine HCl

ethylenediamine tetraacetic acid (EDTA)

N-ethylmaleimide (NEM)

physostigmine sulfate

procaine HCl

sodium iodoacetate (IAA)

tetraethylammonium bromide (TEA)

(2) Inorganic compounds

barium chloride

calcium chloride

mangenous chloride

nickelous chloride

sodium azide

strontium chloride

zinc chloride

RESULTS

I. SPONTANEOUS ACTIVITY IN CA-FREE RINGER SOLUTION

A. Time course of development of spontaneous discharge

The actions of Ca-free Ringer's on electrical activity of ensheathed nerve are shown in Fig. 1. Of particular interest is the delayed appearance of spontaneous activity. After the onset of this activity, there is a gradual increase in amplitude to peak level at 17 to 24 hours. The evoked potential declines at a very slow rate, and even after 22 hours its amplitude is 60% of the initial value. These effects should be compared to the influence of Ca-free Ringer's on the desheathed nerve (Fig. 2). In this case, spontaneous discharge is already evident at 30 seconds and increases to a peak at 3 to 5 minutes. Subsequently there is a relatively rapid decline in discharge amplitude to about 70% of peak value at 10 minutes, and this is followed by a gradual decay until, at about 60 minutes,

a steady state is reached. Coincident with the onset of spontaneous activity, there is a rapid decay of the evoked potential, i.e. during a period of time when spontaneous discharge is increasing, the evoked potential is decreasing. This phase of rapid decay is followed by a much slower decline in amplitude until, as with spontaneous discharge amplitude, a steady state is reached.

The foregoing observations were made on nerves isolated from winter and spring frogs. The nerves isolated from summer frogs do not respond to Ca-free Ringer's in this way. Both the ensheathed and desheathed nerves from summer frogs are remarkably resistant to the absence of external Ca so that the maximal discharge amplitude developed is only about 25% of that observed in nerves from non-summer frogs. About 50% of the "summer nerves" used did not develop spontaneous activity at all.

B. Antagonism of spontaneous discharge by Ca

The amplitude of spontaneous discharge in the ensheathed nerve is very sensitive to Ca concentration in the nerve

bath. This was determined by soaking nerves overnight (16 to 20 hours) in Ca-free Ringer's at 5°C and then testing the effect of various subnormal Ca concentrations on spontaneous activity at room temperature (22-24°C). The results are presented in Fig. 3. The lowest concentration tested, 0.2 mM, produced minimal antagonism of spontaneous activity, and 0.9 mM was required for complete nerve stabilization. In order to reverse the effect of Ca-free solution on evoked spike amplitude, a concentration which completely abolished spontaneous discharge was needed, i.e. 0.9 mM.

C. Antagonism of spontaneous discharge by other divalent cations

It was of interest to determine whether divalent cations other than Ca could produce stabilization in the ensheathed nerve. The testing procedure was similar to that used in the Ca-antagonism studies. After the production of maximal spontaneous activity, the Ca-free Ringer's was replaced by a solution containing 1.8 mM of the test cation, and measurements of spontaneous and evoked potentials were made at periodic intervals for a period of 3 hours. From the curves presented in Fig. 4, the stabilizing effectiveness of the cations tested is as follows: Sr > Zn > Mg > Ni > Ba. The

initial action of Zn and Ni on spontaneous discharge was a slight increase in amplitude; Ba also increased spontaneous activity but not until an initial weak stabilizing effect was produced. Of all the cations tested, only Sr and Mg had a time-action curve similar to that of Ca.

In addition to their stabilizing action, Sr and Zn increased the evoked spike amplitude to the same degree as Ca; the other cations were relatively ineffective in this respect. Perhaps the more important point is that stabilization by divalent cations was never associated with a decrease in the evoked potential.

D. Influence of temperature on spontaneous discharge.

A study was made of the effects of temperature on nerves exposed to Ca-free solution. In these experiments ensheathed nerves were first soaked overnight at 3-5°C in Ca-free Ringer's. Measurements of the amplitude of spontaneous discharge and the evoked spike were then made at various temperatures within the range of 2 to 45°C. This was accomplished by

moving the nerve from one constant temperature bath to another. In Fig. 5 the striking effects of temperature on spontaneous discharge are illustrated, and the biphasicity is quite apparent; raising of temperature up to 20-24°C enhances spontaneous activity but further increases have a stabilizing influence. None of the temperature effects shown in Fig. 5 are accompanied by significant changes in evoked spike amplitude.

The effects of temperature on spontaneous discharge amplitude are shown graphically in Fig. 6. Again, the biphasicity of the temperature effect is obvious, maximal activity occurring at 20 to 24°C. All of the temperature effects are reversible. The temperature of the nerve bath can be changed in either direction by any increment within the range of 3 to 45°C but the effect at any given temperature is remarkably constant and reproducible. The steepness of both phases of the temperature effect is very great, the overall Q_{10} being 4.5.

Another parameter of nerve function that is affected by temperature is absolute refractory period. An increase in

temperature from 3 to 22°C caused about a 40% decrease in the refractory period (as determined by the double nerve shock technique) in normal or Ca-deficient nerve. From 22 to 40°C, a change in temperature which abolishes spontaneous activity, the refractory period decreases still further by about 30%. Therefore, no apparent relationship exists between the influence of temperature on spontaneous activity and the influence on refractory period.

E. Effect of metabolic inhibitors and other pharmacologic agents on spontaneous discharge

The pronounced effects of temperature on spontaneous discharge and the probability that temperature affects nerve metabolism, led to a consideration of the actions of metabolic inhibitors on nerves soaked in Ca-free solution. The compounds tested were sodium azide (NaAz), 2, 4 - dinitrophenol (DNP), iodoacetic acid (IAA) and N-ethylmaleimide (NEM). The results of these experiments are shown in Fig. 7 through 10. A range of concentration of each compound was tested for stabilizing activity, and the minimal effective concentrations (within a period of 205 minutes) are presented in the graphed data. Both DNP and NaAz were relatively specific in their action; at a time when they depressed significantly

the spontaneous discharge, there was no depression of the evoked spike. With IAA and NEM, the depression of spontaneous discharge was accompanied by marked depression of evoked activity.

In addition to the metabolic inhibitors, physostigmine and caffeine were also tested for stabilizing activity. Physostigmine, a potent anticholinesterase, was previously shown to antagonize the depolarizing influence of Ca-free Einger's (Davis and Dettbarn, 1962). In Fig. 11 it is seen that this agent also suppresses spontaneous discharge in concentrations having no effect on the evoked response.

Bianchi (1961) showed that caffeine produces a release of calcium from isolated frog skeletal muscle. It is interesting, therefore, that this agent has a stabilizing action in Ca-deficient frog nerve (Fig. 12).

II. MEASUREMENTS OF THE CA CONTENT OF WHOLE NERVE

A. Effect of normal Ringer solution

Groups of nerves were exposed to a normal Ringer bath (1.8 mM Ca) for a period of time varying from 0.5 to 8 hours, and the total tissue Ca was determined at the end of each soaking period. The Ca content of each group of nerves was compared to that of a control group analyzed for Ca immediately after isolation from the frog. The results of these experiments are summarized in Table 1. After just 0.5 hours in normal Ringer's, the Ca content of the nerve increases significantly, and this increase is maintained for up to 3 hours. The ligated ends of the nerve were cut off just prior to Ca analysis so it is unlikely that the increase of Ca was caused by ions accumulated in the areas of tissue injury.

Other investigators have shown that the concentration of ionized Ca in plasma is lower than that of most *in vitro* bathing solutions (Van Breemen *et al.*, 1966). Accordingly, the enhancement of nerve Ca noted in these experiments may

be caused by a simple concentration gradient favoring the diffusion of Ca into the nerve. Indeed, dialysis of pooled frog serum against various concentrations of Ca showed the ionized serum Ca to be approximately 0.9 mM.

In Table 1, it can also be seen that after 3 hours in normal Ringer's, the nerve Ca returns to control value. After 18 hours in Ringer solution containing 0.9 to 1.8 mM Ca, the nerve Ca was the same as that in control nerves. These results indicate that certain sites for Ca binding in nerve are saturated at the ionized Ca concentration in frog plasma (Table 2).

B. Effect of Ca-free Ringer solution

Using the same procedure as described in the previous section, the influence of Ca-free solution on nerve Ca was determined. The soaking periods varied from 0.5 hours to 6 days. As seen in Fig. 13, there is an initial rapid loss of tissue Ca followed by a slower phase of Ca loss; a steady state is reached at 7-8 hours and is maintained for up to 6 days, indicating a significant fraction of tightly bound nerve Ca.

Of particular interest is a comparison of the time course of Ca loss with the development of spontaneous discharge. From Fig. 1 it is apparent that spontaneous impulses are not observed until after about 4 hours elapse, i.e. after the period of rapid initial loss of nerve Ca. Furthermore, there is no apparent relationship between the enhancement of spontaneous discharge amplitude and the rate of Ca loss after the 4 hour period. For example, the peak spontaneous activity is achieved at a time when nerve Ca remains constant.

C. Effect of high-Ca Ringer solution

Using similar procedures as above, the influence of external Ca concentrations greater than 1.8 mM was determined on nerve Ca. From Fig. 14 it is evident that 10 or 20 mM Ca causes pronounced increases in nerve Ca, indicating the existence of sites for Ca binding that are not saturated by the concentration of ionized Ca in frog plasma or Ringer's.

Fig. 15 is a graph of the relationship between external Ca and nerve Ca after a 4 hour soaking period. In the range of 1.8 to 20 mM external Ca, the relationship is linear, suggesting the existence of a component of nerve Ca that is

in equilibrium with Ca in the external bath.

There is a significant correlation between the change in excitability threshold of the alpha-A fibers and nerve Ca content (Fig. 16).

D. Effect of metabolic inhibitors

If an outwardly directed Ca pump exists in peripheral nerve axons, then metabolic inhibitors should increase nerve Ca. However, neither IAA nor DNP produced significant increases in nerve Ca in concentrations causing marked depression of the evoked spike (Table 3). In fact, DNP produced a consistent slight decrease in nerve Ca.

III. MEASUREMENTS OF CA-45 EFFLUX FROM ENSHEATHED NERVE, DESHEATHED NERVE AND ISOLATED SHEATHS

A. Ca-45 efflux into normal and Ca-free Ringer's

The time course of release of Ca-45 from ensheathed and desheathed nerves into normal or Ca-free Ringer's is shown in Fig. 17 and 18. In each case, the washout curve appears to consist of at least two components. The initial rapid component is conventionally thought to be derived from extracellular sites; the slow component presumably derives from a cellular source.

The time course of decline of nerve radioactivity in ensheathed and desheathed nerves soaking in normal or Ca-free Ringers, is shown in Fig. 19 and 20. In the presence of external Ca there is a high rate of loss of Ca-45 from nerve than in the absence of Ca; but the rate of loss is significantly greater in the desheathed nerve. This same pattern emerges from a study of the rate coefficient curves in Fig. 21 and 22.

From a comparison of the curves which represent washout of Ca-45 from ensheathed nerve into Ca-free Ringer's (Fig. 17, 19, 21) with the time course of development of spontaneous discharge (Fig. 1), it appears that spontaneous firing begins during the slow phase of Ca-45 efflux and is not signalled by a change in slope of the Ca-45 washout curve. The onset of spontaneous firing during a Ca-45 washout experiment is indicated in Fig. 19.

It has long been recognized that the connective tissue sheaths (epineurium and perineurium) are significant barriers to the transport of ions between axons and the nerve bath (cf. Shanes, 1958). The rapid onset of spontaneous discharge in desheathed nerve (Fig. 2) and the relatively high rate of loss of Ca-45 from desheathed nerve, reinforces the view that the sheath plays an important role in the regulation of Ca movements.

The time course of efflux of Ca-45, of the loss of tissue Ca-45, and the rate coefficient curves for isolated sheaths soaking in normal or Ca-free Ringer's, are shown in Fig. 23-25. These graphs show that sheath Ca-45 emerges at a significantly

faster rate into normal than into Ca-free Ringer's. In fact, the rate of loss of Ca-45 from the sheath into normal Ringer's is significantly greater than that from desheathed nerve.

A comparison of the distribution and kinetics of release of Ca-45 in isolated sheath, desheathed nerve and ensheathed nerve leads to some important conclusions; some of the pertinent data are summarized in Tables 4 and 5. From a study of these tables and of the corresponding graphs, it is seen that there is no significant difference between the rates of loss of Ca-45 from ensheathed nerve and isolated sheath into Ca-free Ringer's. This suggests that the connective tissue sheath is the primary rate-limiting barrier to the efflux of Ca-45 from ensheathed nerve into Ca-free Ringer's.

It can also be seen in Tables 3 and 4 that a near equality exists between the rates of Ca-45 release from ensheathed nerve in normal Ringer's and from sheaths or ensheathed nerve in Ca-free Ringers; all of these rates are much slower than that for isolated sheaths in normal

Ringer's. These findings indicate that in ensheathed nerve soaking in normal Ringer's, there is an exchange between axonal and sheath Ca-45. The Ca-45 lost from the axons is probably replaced by Ca-45 from the sheath.

B. Effect of Ca-40 on Ca-45 efflux

In previous studies (Shanes and Bianchi, 1959) it was shown that non-radioactive Ca (Ca-40) causes a prompt and transient release of Ca-45 from frog skeletal muscle if the Ca-40 was added during the slow phase of Ca-45 washout into Ca-free Ringer's. This effect was referred to as Ca-40 - Ca-45 self-exchange. The evidence suggested that the Ca-45 released by Ca-40 was derived from a highly labile membrane source. In the present study, it was determined whether a similar process occurs in frog nerve and nerve sheaths. The tissues were loaded in the usual manner with Ca-45, and the washout of Ca-45 into Ca-free Ringer's was allowed to proceed for 240 minutes in the case of ensheathed nerve, and 150 minutes in the case of desheathed nerve and isolated sheaths; then the Ca-free bath was changed to one containing 1.8 mM Ca-40 (normal Ringer's). The results of these experiments are shown

in Fig. 26-28. In each case, a transient release of Ca-45 occurred.

Changing the nerve bath from normal to Ca-free Ringer's resulted in an immediate decrease in the rate of Ca-45 efflux, an effect also previously observed in frog skeletal muscle by Shanes and Bianchi (1959).

Experiments were also done in which Ca-45 - loaded ensheathed nerves were first permitted to wash out into normal (1.8 mM Ca) Ringer's; then the Ringer's was changed at 240 minutes to solutions containing 5, 10 or 20 mM Ca-40. The results of the experiments with 5 mM Ca-40 are plotted in Fig. 29. The amount of Ca-45 released by each of the aforementioned Ca-40 concentrations was significantly lower than that released by the same concentration of Ca-40 when added to Ca-free Ringer's. It is particularly striking that only 0.01 mM Ca-40 causes a ten times greater release of Ca-45 from a nerve soaking in Ca-free solution than does 5 mM Ca-40 when applied to a normal Ringer medium.

In Fig. 30 the relationship between the concentration of Ca-40 and the magnitude of release of Ca-45 into Ca-free Ringer's is shown graphically. Again note that as little as

0.01 mM Ca-40 causes a significant release of Ca-45 but a saturation is reached at approximately 0.45 mM Ca-40.

C. Effect of temperature on Ca-45 efflux

The interaction between Ca and membrane binding sites should be temperature dependent and, accordingly, the influence of temperature on the slow component of Ca-45 efflux was studied. Some effects of raising or lowering the temperature of the nerve bath are shown in Fig. 31 and 32. It is clear that an increase in temperature, e.g. from 4°C to 20°C, causes a marked release of Ca-45 whereas a decrease in temperature leads to Ca-45 retention. In Fig. 32 the effects of four successive changes of temperature are illustrated; the important finding here is that a change in temperature from 22°C to 41°C, which causes complete antagonism of spontaneous discharge, also produces Ca-45 release. It was also found that the magnitude of Ca-45 released by raising the temperature from 12°C to 22°C (which increases spontaneous activity) was the same as that produced by a change in temperature from 22°C to 32°C (which decreases spontaneous activity).

Interestingly, studies of the effects of temperature on the Ca-40 - Ca-45 exchange reaction, gave somewhat different results. The addition of 0.11 mM Ca-40 at steady-state temperatures of 4°C, 22°C and 32°C, caused maximum and equivalent releases of Ca-45 at the latter two temperatures. It should be remembered that 22°C is also the optimum temperature for spontaneous discharge.

D. Effect of chelating agents on Ca-45 efflux

It was found that EDTA produces Ca-45 release from isolated ensheathed or desheathed nerve (Fig. 33a) and also from isolated sheaths (Fig. 33b). In the range of 2 to 10 mM EDTA, there is a linear relationship between concentration and the peak release of Ca-45 from these tissues. The effect of any of these concentrations of EDTA is transient, i.e. despite the persistence of EDTA in the bath, the increase in Ca-45 efflux is short-lived. In confirmation of previous work done in this laboratory (Kuperman et al., 1967), the nucleoside phosphates ATP and AMP were also shown to increase the rate of Ca-45 efflux in isolated nerve. At 10 mM, the peak effect of ATP was greater than that of AMP.

E. Effect of foreign external divalent cations on Ca-45 efflux

At 1.8 mM, a variety of divalent cations increased the rate of Ca-45 efflux from the isolated nerve. As seen in Fig. 34, Ca and Sr are equipotent as Ca-45 releasers, and the order of potency of the entire series is Ca = Sr > Ba > Mg = Zn = Ni > Mn. On closer inspection, it appears that Ca, Sr and Ba form one group of relatively potent releasers of Ca-45 while the remaining cations form a weaker group.

F. Effect of external univalent cations on Ca-45 efflux

Whereas a variety of divalent cations were shown to be effective releasers of Ca-45, the monovalent cations K^+ and H^+ are relatively ineffective. Some of the results obtained with K^+ are shown in Fig. 35. It is seen that even isotonic KCl, which exerts a strong depolarizing influence, causes a minimal effect on the rate of Ca-45 efflux; the amount of Ca-45 released by 116 mM KCl is even less than that released by 1 mM $CaCl_2$.

The influence of H^+ was studied by varying the pH of the nerve bath from 7.2 to 4.3. This increase in H^+ concentration (obtained by the addition of HCl to the nerve bath) had a negligible effect on the rate of Ca-45 efflux (Fig. 35).

G. Effect of pharmacologic agents on Ca-45 efflux

It is well known that TEA produces spontaneous repetitive discharge in isolated frog nerve (Cowan and Walter, 1937). The resemblance between this effect and that of Ca-deficiency is so great, that it seems reasonable to suppose that the TEA cation produces a displacement of Ca from membrane binding sites. This suggestion is not supported, however, by the finding that in concentrations causing spontaneous firing (10 mM or greater), TEA does not affect the rate of release of Ca-45 from ensheathed or desheathed nerve. Furthermore, these same concentrations of TEA have no effect on the Ca content of ensheathed nerve (Table 6).

It has recently been proposed that the depressant action of procaine and other local anesthetics on peripheral axons is associated with the displacement of membrane Ca. (Blaustein and Goldman, 1966). This hypothesis is based on the finding that high external Ca antagonizes the depressant effect of procaine on the inward current in voltage-clamped lobster axons; high Ca also antagonizes the anesthetic effect of procaine on the action potential itself. In the present experiments, it was found that procaine increases the rate of Ca-45 efflux from frog ensheathed or desheathed nerve and from isolated sheaths (Fig. 36 a, b). Interestingly, the more potent local anesthetic, dibucaine caused a more enduring release of Ca-45 (from either the nerve of isolated sheath) than equimolar concentrations of procaine. In further support of the interaction between local anesthetics and Ca, it was found that procaine causes a small but significant loss of Ca from the whole nerve (Table 6).

The influence of caffeine on Ca-45 efflux was studied because the compound was found to antagonize spontaneous discharge in Ca-deficient nerve. Caffeine does cause a relatively small but definite release of Ca-45 from isolated

ensheathed and desheathed nerve (Fig. 37).

It was previously stated that the metabolic uncoupling agent DNP stabilizes the Ca-deficient nerve and also causes a small decrease of total nerve Ca. Accordingly, this agent was also tested on Ca-45 efflux. At 10 mM, DNP causes a significant increase in the rate of Ca-45 efflux (Fig. 38). This type of action was also shown by Koketsu (1965) in frog skeletal muscle and was attributed to dissociation of bound Ca from membrane lipoprotein.

H. Effects of electrical stimulation on Ca-45 efflux

In order to determine the influence of electrical stimulation on Ca-45 efflux from ensheathed nerve, the nerve was placed across bipolar platinum stimulating electrodes in the collecting fluid. A voltage high enough to insure excitation of the alpha-A fibers was applied. As can be seen in Fig. 39, a release of Ca-45 was produced by the application of electrical pulses, the intensity of which was related to the stimulus frequency. Because it

was suspected that this effect was caused, at least in part, by junctional potentials at the point of contact between the electrodes and the nerve, another procedure was adopted. The nerve was placed in a two-compartment chamber, one for stimulation and the other for fluid collection. Mineral oil, isotonic sucrose or normal Ringer's was placed in the stimulation compartment, and the maximal voltage (for alpha-A fibers) was applied at varying frequencies. In some experiments a relatively small release of Ca-45 into the collection fluid was obtained; in other cases no effect of electrical stimulation was seen. It would appear that nerve impulses are associated with a small, highly variable, and unreliable influence on the rate of Ca-45 efflux into Ca-free Ringer's.

IV. MEASUREMENTS OF EXTRACELLULAR SPACE AND EXCHANGEABLE CA IN ENSHEATHED NERVE

In order to calculate the various components of nerve Ca, both ionized and bound, the extracellular space was determined. Sucrose and inulin, labeled with C-14, were used as the impermeant solute molecules, and their volume of distribution was measured after overnight equilibration. The sucrose space was found to be (ml. per gram wet weight) 0.429 ± 0.0106 and the inulin space was 0.311 ± 0.0080. These values differ significantly ($P < 0.001$). It has been reported that sucrose does indeed penetrate intracellular sites and/or binds to surface membranes; if this is the case then space determinations with sucrose would be expected to yield misleadingly high values (Bozler, 1961). Therefore, the inulin space value will be viewed as the truer measurement.

The radioactivity washout curves of either C-14 labeled sucrose or inulin contained more than one component, suggesting that the extra-axonal space (assuming that inulin, at least, does not penetrate axons) consists of more than one compartment.

The presence of myelin and connective tissue sheaths in the nerve bundle almost certainly accounts for the complexity of the washout curves.

From inspection of the washout curves of radiocalcium and radioinulin into Ca-free Ringer's, it was calculated that the extracellular space is emptied in approximately 60 minutes; this is assuming that the initial rapid component of radioactive washout derives from extracellular (or at least extra-axonal) spaces. During this period of rapid washout into Ca-free Ringer's, there is a loss of 0.7 mM Ca per kilogram of wet weight. Utilizing the value obtained for space and the known amount of ionized Ca in the Ringer solution (1.8 mM), the extracellular ionized Ca in the ensheathed nerve is calculated to be 0.56 mM per kilogram wet weight. Thus, the loosely bound Ca fraction, exchangeable with monovalent cations of the Ca-free Ringer's, is 0.14 mM per kilogram wet weight. After soaking in a Ca-free Ringer solution for 6 days, there remains a bound (non-exchangeable) Ca fraction of 0.6 mM per kilogram wet weight. If this value plus the amount of Ca initially effluxing into the Ca-free Ringer's (0.7 mM per kilogram wet weight) is

subtracted from the total nerve Ca (2 mM per kilogram wet weight), a value of 0.7 mM is obtained. This represents approximately the total amount of cellular calcium that is exchangeable with the medium.

DISCUSSION

EVIDENCE FOR EXCHANGEABLE CALCIUM IN FROG NERVE.

Evidence obtained from previous experiments with muscle and nerve suggests that a membrane-bound labile fraction of calcium participates in excitation phenomena (Frankenhaeuser and Hodgkin, 1957; Brink, 1954; Nayler, 1965; Koketsu, 1965). In the present investigation, experiments were designed to determine and characterize a labile fraction of calcium in frog sciatic nerve.

Results obtained from space studies, studies of the distribution of calcium in the nerve bundle and of the efflux of calcium into calcium-free Ringer's, suggest the presence of a labile calcium component. The early efflux into calcium-free Ringer's exceeds that calculated for extracellular ionized calcium. This component of calcium must therefore be a labile fraction of calcium which is probably exchangeable with various monovalent cations of the medium (Shanes and Bianchi, 1959; Koketsu, 1965).

It is thought that "the dissociation of calcium ions takes place only when calcium is displaced by another cation" (Koketsu, 1965). Since only monovalent cations are present in a calcium-free Ringer's and since it has been demonstrated in both phospholipid films and cytoplasmic membranes that sodium, potassium and hydrogen ions compete with calcium for binding sites (Koketsu et al., 1964; Palmer and Schmitt, 1941), it seems reasonable to assume that the calcium fraction lost in a calcium-free medium is freely exchangeable with the monovalent cations. That this fraction is also exchangeable with calcium itself is evident from the fact that it becomes labelled merely when the nerve is soaked in Ca-45 Ringer's. It is Koketsu's view that this fraction of calcium must be the one involved in excitability, because an exchange between sodium and calcium is a basic tenet of his hypothesis of excitation (1965).

Previous studies have shown that a component of muscle calcium is rapidly released upon addition of external calcium (Shanes and Bianchi, 1959). It was suggested that this calcium

is loosely bound to membrane surfaces. When paired intact frog sciatic nerves soaked overnight in a Ca-45 Ringer are allowed to efflux into (a) a normal Ringer solution as opposed to (b) a calcium-free Ringer solution, there is a more rapid emergence of Ca-45 in (a). If loaded nerves are immersed in a normal Ringer solution for four hours and then switched to a calcium-free solution, there is an immediate <u>decrease</u> in the rate of efflux of Ca-45. The reverse experiment, i.e. switching from calcium-free to normal Ringer's after four hours, shows a rapid release of Ca-45. These experiments suggest the existence of a loosely bound calcium fraction, presumably located in axon surfaces, that is exchanging primarily with calcium in the nerve bath and to a limited extent with monovalent cations. A concentration of potassium as high as 116 mM causes a relatively slight increase in the rate of efflux of Ca-45 from ensheathed nerve, and a high concentration of hydrogen ions (pH 4.3) has no effect at all. That this self-exchangeable calcium fraction is membrane- or loosely-bound, can be reinforced

by other experiments in which it is shown that when high calcium concentrations are added to loaded nerves effluxing into normal Ringer solution, little additional Ca-45 emerges. It would thus appear that most of this particular calcium fraction exchanges with external calcium during the early rapid phase of Ca-45 washout.

Quantitative analysis of the results of experiments on exchange between Ca-45 and Ca-40 in ensheathed nerve reveals that there is saturation of these exchangeable sites at an external concentration of calcium between 0.45 mM and 1.8 mM. A low saturation concentration for self-exchangeable sites has also been reported for muscle (Shanes and Bianchi, 1959). It should be noted at this point that a minimal calcium concentration of 0.4 mM not only seems to saturate such self-exchangeable sites, but is also about the minimal concentration required to antagonize spontaneous discharge in calcium-deficient nerve.

Since a labile calcium component can be unmasked by the addition of external calcium, it was of interest to

determine whether other divalent cations could exchange with this component. Addition of various divalent cations to nerve labelled with Ca-45, each in a concentration of 1.8 mM, caused a rapid and transient release of calcium into the medium. The order of effectiveness on Ca-45 release was as follows: calcium = strontium > barium > magnesium = zinc = nickel > manganese; calcium, strontium and barium form one group of relatively potent calcium releasers and the other ions form another group of weak releasers. These findings seem to agree closely with the work of Nayler (1965) on toad cardiac muscle. She noted that barium and strontium released about as much Ca-45 as calcium did, while zinc, magnesium and nickel released smaller amounts. She interpreted these results to mean that barium and strontium release calcium from cellular stores, while the other cations are restricted to the rapidly-exchanging fraction of calcium. In her preparation, barium and strontium could support and activate the contractile mechanism, while the other ions could not. Another interpretation of these results seems

possible, as will be discussed later.

Gilbert and Fenn (1957) showed that, in frog skeletal muscle, EDTA removes more tissue calcium than calcium-free Ringer's. The present experiments with Ca-45 labelled nerves, showed a rapid and transient release of Ca-45 after addition of EDTA. This was demonstrated for intact nerves as well as desheathed nerves and sheaths. Two other complexing agents, AMP and ATP (Hoffman, 1962), produce similar results. Since these various organic anions are not likely to penetrate the cell membrane (Bianchi, 1965; Hoffman, 1961), the concept of a labile membrane-bound calcium fraction is reinforced. The calcium-releasing actions of these agents are probably not associated with removal of extracellular calcium because the effect of a calcium-free Ringer's on a nerve soaking in normal Ringer's, is to cause a <u>decrease</u> in the rate of calcium release.

Temperature changes should not only exert an effect on calcium binding in the membrane, but also serve to test for the presence of a labile membrane-bound calcium fraction.

Indeed, increases in temperature produced marked increases in the rate of Ca-45 release into calcium-free Ringer's; a decrease in temperature produced a retention of calcium. This phenomenon could be demonstrated repeatedly on the same preparation. Equal 10-degree increments in temperature, e.g. from 12 to 22°C and from 22 to 32°C, produced approximately the same release of radioactivity. It is noteworthy that when various self-exchange experiments were carried out with 0.11 mM Ca-40 Ringer's at 4, 22 and 32°C, the greatest calcium release was obtained at 22°C (room temperature).

RELATIONSHIP BETWEEN MOVEMENTS OF CALCIUM AND EXCITABILITY.

A reduction in external calcium produces spontaneous activity and a reduced threshold for the evoked spike. The present experiments point to the presence of two labile, membrane-bound, calcium components, one exchangeable with monovalent cations and the other primarily with calcium itself. It was therefore of interest to ascertain the nature of the relationship between calcium movement and excitability in calcium-free media. It was shown that spontaneous activity

develops only after total tissue calcium is reduced to about 50% of that in normal Ringer's. It is understood that the cellular calcium lost in calcium-free medium is monovalently exchangeable calcium (as discussed above). Both the amplitude and frequency of spontaneous discharge increase as total tissue calcium is further reduced to 30% of normal; but despite no further loss of calcium, the spontaneous discharge continues to increase to maximum and is maintained for many hours in the ensheathed nerve at a maximal level.

It is interesting that spontaneous discharge continues to increase during a period when there is no further calcium loss. This could be interpreted to suggest that the loss of calcium, although initially required to trigger spontaneous activity, is not the only event underlying maintenance and continued development of spontaneous firing. Indeed, Abood et al. (1964) found that a variety of organic and inorganic phosphates are lost from nerve and muscle in calcium-free solution. There is an especially significant loss of ATP so that Abood focuses on the role of ATP or a calcium-ATP macromolecular membrane complex as being primarily implicated

in excitation phenomena. In support of the role of ATP as an important constituent in the excitable membrane, is the finding by Kuperman et al. (1967) that ATP stabilizes the calcium-deficient nerve. On the other hand, it must be emphasized that the simple addition of calcium (or some other divalent cation) to the nerve bath will abolish all signs of spontaneous firing. It is therefore possible to make another interpretation of these results as follows: the removal of the monovalently exchangeable calcium fraction labilizes the self-exchangeable component, which is more intimately structured in and concerned with the architecture of the excitable membrane, thus creating the disruptions seen as electrical spontaneous oscillations. Other investigators have referred to a calcium storage site which must be depleted before changes in cell function are revealed. It has also been shown that the mobilization of calcium in one cellular site can be inhibited by calcium binding at a second, perhaps more superficial site (Hurwitz, 1967).

At a time when maximum spontaneous firing occurs, the evoked spike is reduced in amplitude, especially in the

desheathed nerve. This suggests the existence of a relationship between evoked spike amplitude and a calcium component that is exchangeable with monovalent cations. Support for this suggestion derives from experiments showing that the addition of calcium or other divalent cations to a calcium-free Ringer's reverses the depression in spike amplitude. The action of procaine, which abolishes the spike in appropriate concentrations and also releases self-exchangeable calcium (Feinstein, 1963; Kuperman et al. 1968) and certain metabolic inhibitors which also abolish the spike and release calcium, implicate another component of calcium that is involved in evoked membrane depolarization.

Small increases in temperature induce a release of radiocalcium from the frog nerve, and small decreases in temperature produce a retention of radiocalcium. Likewise, in the range of temperature between 0 and 24°C, spontaneous discharge amplitude is linearly related to temperature. This suggests a direct relation between calcium movement and spontaneous activity. However, change in temperature from 22 to 42°C, which causes complete block of spontaneous discharge, also causes a release of radiocalcium. Furthermore,

the amount of radiocalcium released by a change from 12 to 22°C does not differ significantly from the amount released by a change from 22 to 32°C. This occurs despite the fact that one change in temperature causes a tripling in spontaneous discharge amplitude, while the other causes about 50% decrease in spontaneous activity. These experiments, therefore, demonstrate a component of nerve dissociable calcium that is sensitive to temperature; but there seems to be no relationship between this temperature-sensitive calcium and the intensity of spontaneous discharge in calcium-deficient nerve.

The influence of temperature on the release of Ca-45 by nonradioactive calcium was also studied. The results showed that maximum release was produced at a bath temperature of 22°C, which is within the range of temperature (20-24°C) at which maximum spontaneous activity occurs. On the basis of this kind of information, it appears that a relationship exists between spontaneous activity and the movement of a self-exchangeable component of calcium.

The primary effect of temperature may be exerted on noncovalent bonds in membrane macromolecules with consequent changes in molecular configuration. On this basis, temperature would effect calcium release from the self-exchangeable sites and spontaneous activity because both are dependent on molecular structure of membrane macromolecules.

The data obtained from studies of the relationship between the time course of development of spontaneous discharge and changes in calcium content and rate of Ca-45 efflux from nerves soaked in calcium-free solution, the closeness of the concentration of calcium required to saturate self-exchangeable sites and to antagonize spontaneous activity, and the similarity of temperature effect on spontaneous activity and on Ca-45 - Ca-40 exchange - all of these results can be used as a basis of the following proposed mechanism of spontaneous discharge: the absence of external calcium leads to a loss of membrane calcium from the monovalently exchangeable

component. This labilizes the self-exchangeable component so that at any given moment in time, fewer of these calcium-binding anionic sites are occupied by calcium, and this alters the molecular architecture of the membrane such that the permeability to sodium is increased.

Some clues to the location and physico-chemical properties of the self-exchangeable calcium binding sites derive from studies of the release of Ca-45 by foreign divalent cations. One interpretation of the data is based on the concept of a "superficial" and "deep" calcium store (Nayler, 1965). Thus, the relatively potent group of calcium releasers - calcium, strontium, barium - may act at a deep site while the weaker group - magnesium, zinc, nickel, manganese - acquire access only to more superficial binding sites. As the basis for an alternative explanation of the data, it should be considered that <u>within each group</u> of calcium releasers there is a good correlation (with the exception of magnesium) between releasing potency and complexing affinity to chelators like EDTA, citrate and oxalate (Albert, 1961; Martell and Chaberek, 1959). Interestingly, the group of relatively weak calcium releasers consists of transition metals, which form more stable

complexes with chelating agents than the more potent group of releasers which consists of alkaline earth cations. This may reflect differential affinities for different ligand groups. For example, manganese, zinc and nickel cations prefer nitrogen donor groups while calcium, strontium and barium have the stronger affinity for oxygen groups (Martell, 1961; Williams, 1961). It is interesting to note that the best fit for the transition metal data is with a sulfur-containing group (Williams, 1961). Accordingly, it seems reasonable to consider the possibility that there are at least two binding sites for the so-called self-exchangeable component of calcium; one site can bind alkaline earth cations, including calcium; the other can bind transition metals.

The effect of an external foreign divalent cation on spontaneous discharge of calcium deficient nerve should depend not only on the stability constant of cation binding but also on the conformational change which this binding induces in appropriate membrane macromolecules. With the exception of strontium and magnesium, all the divalent cations

tested not only released calcium and antagonized spontaneous firing but they also were observed to increase spontaneous discharge, if only transiently. This may be caused by the induction of a conformational change that favors the maintenance and enhancement of spontaneous discharge.

The release of calcium from certain membrane binding sites by the addition of calcium or another divalent cation to the nerve bath or by calcium dissociating from other binding sites, may not be a simple exchange process. Abood (1965) suggests that calcium catalyzes the disruption of bonds between calcium and its ligand, and this can account for the intense release of Ca-45 by very low concentrations (0.01 mM) of external calcium. Alternatively, the action of a divalent cation at one site on a membrane macromolecule may induce a conformational change such that calcium dissociates from another site; this may be likened to an allosteric effect.

EFFECTS OF DRUGS AND METABOLIC INHIBITORS

An important theme that emerges from the foregoing discussion is that the mere loss of dissociable calcium

into a calcium-free medium is insufficient for initiation and maintenance of spontaneous firing; the presence of some component of calcium, probably a membrane-bound self-exchangeable component, is also needed. The relationship between this calcium component and spontaneous discharge receives support from studies of the action of stabilizing drugs. Compounds that depress spontaneous discharge in concentrations that do not affect the evoked spike, cause a release of nerve calcium; these compounds include local anesthetics, caffeine and DNP.

The calcium releasing action of procaine and tetracaine in frog desheathed nerve and skeletal muscle has been reported (Kuperman et al., 1968), and it was observed that the more potent local anesthetic, tetracaine, produced a more intense and enduring calcium release than procaine.

In the present experiments it was shown that the more potent agent, dibucaine, is more effective as a calcium releaser than procaine. Accordingly, it seems reasonable to suggest that the calcium release caused by local anesthetics

is somehow related to the effects of these drugs on excitability of normal or calcium-deficient nerve.

The calcium releasing action of caffeine is interesting in that even very large concentrations do not depress the evoked spike. Thus, the effect on calcium efflux seems more clearly related to a stabilizing action, i.e. to antagonism of spontaneous firing. In skeletal muscle, caffeine also causes a calcium release, even after the action of EDTA (Bianchi, 1961), and it releases calcium from membrane and subcellular fragments of both skeletal and cardiac muscle (Bianchi, 1961; Frank, 1962; Herz and Weber, 1965; Nayler, 1966).

The calcium releasing action of DNP is not associated with depression of the evoked spike, and concentrations of DNP that abolish spontaneous firing do not simultaneously affect the evoked spike. It may be argued, therefore, that the effect of DNP on calcium efflux is not related to its well-known uncoupling effect on oxidative phosphorylation; the latter effect is probably intimately associated with membrane depolarization and depression of evoked spike.

Another uncoupling agent, NaAz, also has a highly specific stabilizing action. The sulfhydryl inhibitor, N-EM, and the inhibitor of glycolysis, IAA, are not true stabilizing agents for they depress both the spontaneous firing and evoked spike simultaneously.

Considering the number of compounds that release nerve calcium, it is well to emphasize at this time that this property is not associated with all drugs. For example, the very potent depressant of excitation, tetrodotoxin, lacks a calcium releasing action (Kuperman et al., 1968), and so does TEA. The lack of a TEA effect on calcium efflux is especially interesting for TEA actually produces the same type of spontaneous discharge in frog nerve as does calcium deficiency. Furthermore, the TEA-treated nerve shows the same temperature sensitivity as does a calcium deficient nerve. The apparent paradox disappears, however, with the recognition that some component of nerve calcium is required for the maintenance of spontaneous discharge, and if TEA did cause release of this calcium, it would probably

have a stabilizing rather than a labilizing action on calcium deficient nerve. In addition to the inability of some drugs to affect the rate of calcium efflux from nerve or the calcium content of nerve, it is also important to note that the highly depolarizing medium, isotonic KCl, produces a negligible calcium release compared to that caused by stabilizing drugs discussed above or even by calcium itself.

In general, the foregoing pharmacologic data support the hypothesis, previously based on the results of experiments with Ca-45 and measurement of calcium content, that a loosely-bound component of nerve calcium supports spontaneous discharge in calcium deficient nerve. This hypothesis is also in accord with experiments carried out on single frog myelinated axons (Frankenhaeuser, 1957) showing that complete removal of external calcium produces inexcitability rather than spontaneous firing. Also, Rosenberg and Bartels (1967) point out that isolated giant axons of squid do not exhibit spontaneous firing in calcium-free Ringer's but that the addition of calcium to the medium triggers repetitive impulses.

EFFECTS OF ELECTRICAL STIMULATION

A relationship between nerve or muscle excitation and the influx and/or efflux of calcium, has been previously reported (Hodgkin and Keynes, 1957; Koketsu and Miyamoto, 1961; Shanes, 1958). In the present experiments, high-frequency electrical pulses produced only a small and rather inconsistent release of calcium if the stimulated segment of nerve was deliberately separated from the length of nerve located in the effluent solution. Nerve depolarization by isotonic KCl also caused relatively small increases in the rate of calcium efflux. Similar results were obtained with rat smooth muscle (van Breemen and Daniel, 1966). At the present state of our knowledge about membrane calcium, it is difficult to explain how two types of conditions - one which stabilizes the calcium deficient nerve and the other which causes membrane depolarization - can both be associated with the release of calcium. Are two different components of calcium involved or are there many factors in addition to calcium release that are involved in the molecular basis of

membrane stabilization and depolarization? Perhaps depolarizing stimuli release only that component of calcium that also rapidly and initially dissociates from membrane binding sites in calcium-free Ringer's but the self-exchangeable component remains unaffected.

THE REGULATION OF CALCIUM MOVEMENT

Role of the sheath. The striking difference in time to onset of spontaneous firing between calcium deficient ensheathed and desheathed nerves, is strong evidence that the connective tissue sheath has an important role in the regulation of calcium movement between the axons, the extra-axonal spaces and the nerve bath. The role of the sheath as a diffusion barrier has been argued for a long time (Geng and Gerard, 1930; Lorente de Nó, 1950; Crescitelli, 1951; Krnjevic, 1954; Lehman, 1953), and there seems little doubt that the sheath does indeed slow the rate of ion movement between axons and the nerve bath (Shanes, 1958). On analysis of the efflux data, the following becomes apparent: the fastest rate of loss of calcium into calcium-free Ringer's occurs

in the desheathed preparation; the kinetics of calcium efflux from ensheathed nerve into calcium-free or normal Ringer's is very similar to the kinetics of calcium efflux from the sheath into calcium-free Ringer's; percentage-wise, more sites for calcium self-exchange seem to exist in the sheath than in the other tissues, and more self-exchange sites are in the desheathed nerve than in the intact nerve. Additional determinations of calcium content (unpublished observations) showed that the sheath has a great affinity for calcium and has about five times as great a calcium concentration as desheathed nerve. It seems clear from the data that the sheath governs the efflux of calcium from ensheathed nerve into calcium-free Ringer's, and the possibility also exists that a continual exchange of calcium occurs between nerve and sheath in ensheathed nerve. The remarkable retention of calcium by the sheath in a calcium-free medium and the possibility that the sheath both binds and exchanges with calcium in the ensheathed nerve, denotes this tissue as a prime regulator of movement of membrane calcium.

Role of metabolism. A metabolically-dependent outwardly-directed calcium pump has been postulated for the squid giant axon (Hodgkin and Keynes, 1957); the concentration of ionized calcium in the axoplasm is relatively low despite the fact that the membrane is permeable to calcium and that the electrochemical gradient favors calcium influx. An ATP - dependent calcium pump has been found in sarcotubular elements of skeletal muscle (Weber et al., 1963) and in mitochondira (Brierley et al., 1963) but a similar pump for cell membranes has so far eluded investigation (Weber, 1966). The present measurements of calcium content in frog nerve in the presence of metabolic inhibitors have also proved negative. Both IAA and DNP, in concentrations greater than are needed to stabilize calcium deficient nerve, do not affect calcium content.

SUMMARY

The purpose of these experiments was to correlate calcium content and fluxes in frog peripheral nerve axons soaking in calcium-free Ringer solution with simultaneous changes in electrophysiological activity. Of particular interest was the relationship between calcium movements and the development and maintenance of spontaneous discharge in such nerves. To this end, the following measurements were made on isolated ensheathed nerves soaking in calcium-free Ringer's: (1) the time course of development and decay of spontaneous discharge; (2) the loss of total cellular calcium; (3) the rate of loss and the rate of efflux of radiocalcium (Ca-45) from isolated nerve into Ca-free Ringer's; (4) the effect of ions (including calcium itself), drugs, metabolic inhibitors and temperature on the electrophysiological parameters, Ca-45 washout and calcium content; (5) the extracellular space of isolated frog sciatic nerve.

The results of these experiments indicate that frog nerve axons contain two components of labile, so-called exchangeable, calcium, and presumably both are membrane-bound. One component is exchangeable with external monovalent cations and calcium while the other is exchangeable primarily with calcium; this latter component is referred to as "self-exchangeable". The self-exchangeable fraction is saturated at relatively low external calcium concentrations, appears to have at least two different binding sites, and is mobilized by various drugs, metabolic inhibitors, calcium complexers and increases in temperature. Those chemical and physical agents that stabilize the calcium-deficient nerve, i.e. abolish spontaneous firing without simultaneously depressing the evoked spike, cause a release of nerve calcium. This suggests that some fraction of axonal calcium must be present in order for spontaneous discharge to be developed and maintained in a calcium-free medium.

A nerve soaking in calcium-free Ringer's loses about

one-half of its total cellular calcium prior to the appearance of spontaneous firing. At a time when spontaneous firing begins and increases to a maximum intensity, there is relatively little additional loss of cellular calcium. This indicates that an initial loss of calcium is not sufficient, by itself, to initiate spontaneous firing. It is proposed that the loss of this calcium labilizes the self-exchangeable calcium component so that at any given moment in time, fewer of these binding sites are occupied by calcium; this, in turn, leads to an alteration of the membrane's molecular architecture such that sodium permeability is enhanced.

The role of the connective tissue sheath surrounding the nerve bundle has also been studied in this investigation, particularly with a view towards understanding the function of the sheath as a regulator of calcium flux between axons and nerve bath. The finding that calcium deficient desheathed nerve develops maximum spontaneous firing within five minutes and ensheathed nerve does not even begin to show this effect

until after about three hours, strongly implicates the sheath as a regulator of calcium content of nerve axons. It was shown that the sheath has a strong affinity for calcium but that this sheath-bound calcium is also released by the same agents as those which release calcium from the desheathed or ensheathed nerve. Particularly effective in releasing calcium from the sheath is calcium itself. In a calcium-free medium, the sheath has a remarkable ability to retain calcium. All of the available data lead to the suggestion that in the ensheathed nerve soaking in calcium-free medium, there is self-exchange between axonal calcium and sheath calcium, and it is this process that delays the appearance of spontaneous firing in the ensheathed nerve.

TABLE 1

Effect of normal Ringer solution on calcium content of ensheathed nerve

Time (hrs.)	Ca content (mM/kg wet wgt. ± S.D.)
0	2.0 ± 0.2
½	2.7 ± 0.3
1	3.1 ± 0.6
2	3.2 ± 0.5
3	2.8 ± 0.5
4	2.2 ± 0.6
5	2.0 ± 0.1
6	1.9 ± 0.1
8	2.0 ± 0.1

TABLE 2

Effect of low-calcium Ringer's on calcium content of ensheathed nerve after 18 hours treatment

Ca in Ringer's (mM)	Ca content (mM/kg wet wgt. ± S.D.)
1.0	1.9 ± 0.1
1.2	1.8 ± 0.5
1.3	1.8 ± 0.4
1.8	2.3 ± 0.6

TABLE 3

Calcium contents of ensheathed nerves after treatment with DNP[1] or IAA[2]

	Nerves	DNP mM/kg dry wgt	Na IAA mM/kg dry wgt
1	Control	7.57	8.89
	Test	7.16	7.04
2	Control	6.76	8.10
	Test	5.71	7.20
3	Control	7.24	7.54
	Test	6.88	8.32
4	Control	6.80	6.25
	Test	5.97	8.04
5	Control	7.56	6.47
	Test	7.39	7.01
6	Control	8.33	7.28
	Test	7.74	8.49
7	Control	7.63	8.39
	Test	7.37	8.28
8	Control	7.12	7.32
	Test	7.89	7.16
9	Control	6.60	6.44
	Test	6.88	7.15
10	Control	6.68	
	Test	6.56	
11	Control	6.55	
	Test	6.45	
Significance[3]		$P > 0.05$	$P > 0.05$

1 1.0 mM in normal Ringer at pH 6.0; 1.5 hours treatment

2 10 mM in normal Ringer at pH 7.2; 4 hours treatment

3 Calculated according to Paired T Test.

TABLE 4

Radiocalcium remaining in tissue (% initial) at the end of 6 hours immersion in normal or Calcium-free Ringer's

Data obtained from 6-12 nerves or sheaths

Tissue	Normal Ringer's	Ca-free Ringer's
Ensheathed nerve	23.8%	27.9%
Desheathed nerve	5.7%	13.3%
Sheath	3.7%	24.6%

TABLE 5

Comparison of rate coefficients at different times during efflux of radiocalcium in normal or calcium-free Ringer's

Data obtained from 6-12 different nerves or sheaths

Time (min.)	Tissue	Normal Ringer's	Ca-free Ringer's
100	Ensheathed nerve	0.36	0.35
	Desheathed nerve	0.80	0.56
	Sheath	1.17	0.38
200	Ensheathed nerve	0.28	0.26
	Desheathed nerve	0.58	0.39
	Sheath	0.45	0.40
360	Ensheathed nerve	0.22	0.17
	Desheathed nerve	0.45	0.22
	Sheath	0.26	0.20

TABLE 6

Calcium contents of ensheathed nerves after treatment with TEA[1] or procaine[2]

	Nerves	TEA mM/kg dry wgt	PROCAINE mM/kg dry wgt
1	Control	6.09	7.17
	Test	7.47	6.85
2	Control	7.74	7.24
	Test	7.56	7.73
3	Control	7.14	7.92
	Test	8.32	6.51
4	Control	6.81	7.18
	Test	7.38	6.61
5	Control	7.23	7.25
	Test	7.67	6.01
6	Control	7.37	7.51
	Test	7.18	7.80
7	Control	8.69	7.39
	Test	9.81	6.67
8	Control	8.00	7.30
	Test	7.54	6.37
9	Control	7.50	8.00
	Test	7.81	6.49
10	Control		7.53
	Test		6.92
11	Control		6.95
	Test		6.77
12	Control		6.68
	Test		6.72
Significance[3]		$P > 0.05$	$P < 0.05$

1 10 mM in normal Ringer at pH 7.2; 1 hour treatment

2 20 mM in normal Ringer at pH 7.2; 1 hour treatment

3 Calculated according to Paired T Test

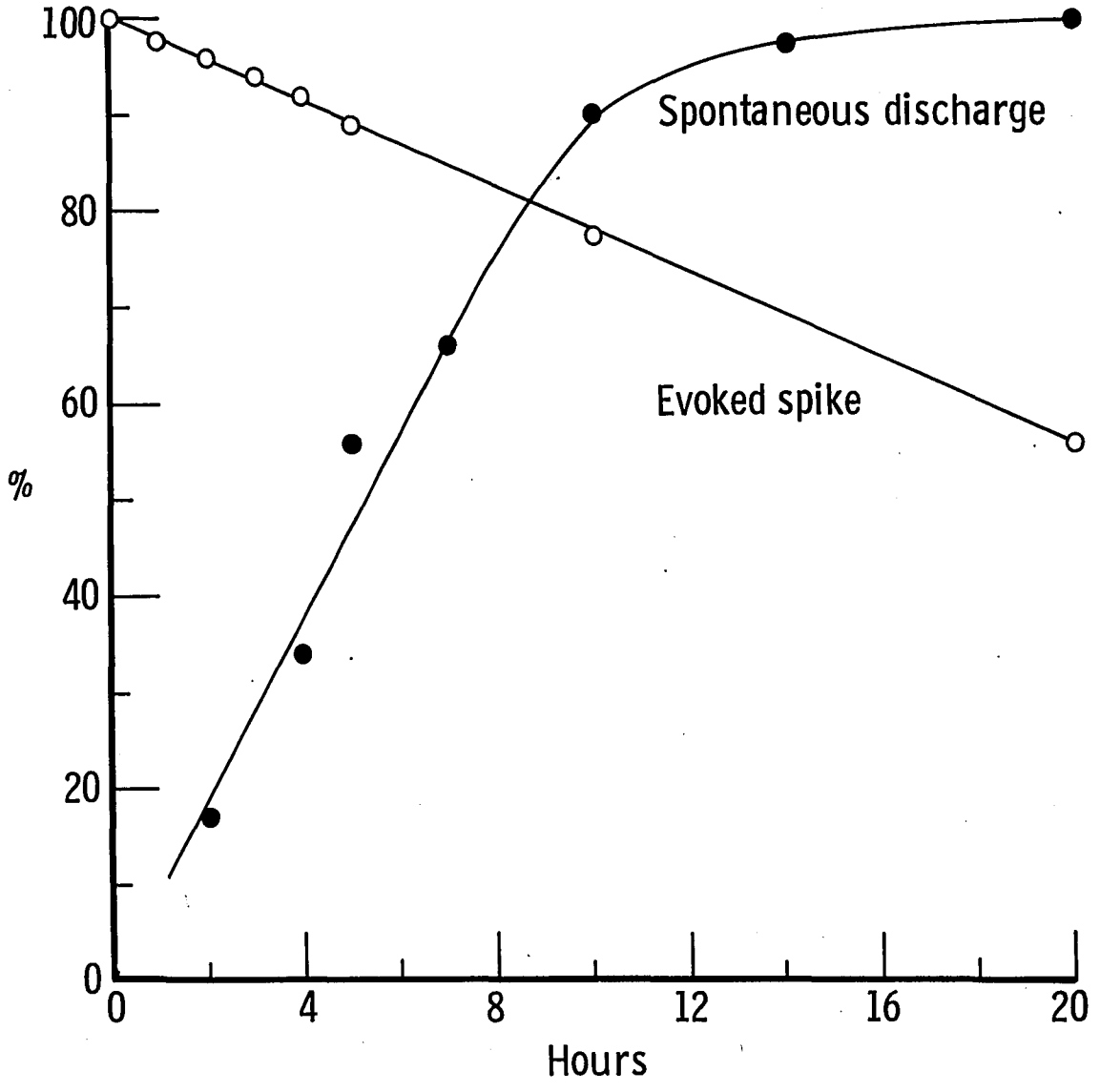


Figure 1. The time course of spontaneous discharge and the changes in amplitude of the evoked alpha-A fiber spike in ensheathed nerves soaked in Ca-free Ringer. Each point is an average of 6-11 separate experiments. Spontaneous discharge amplitudes were calculated as percentages of maximum effects; evoked spike amplitudes were calculated as percentages of spike heights in normal Ringer.

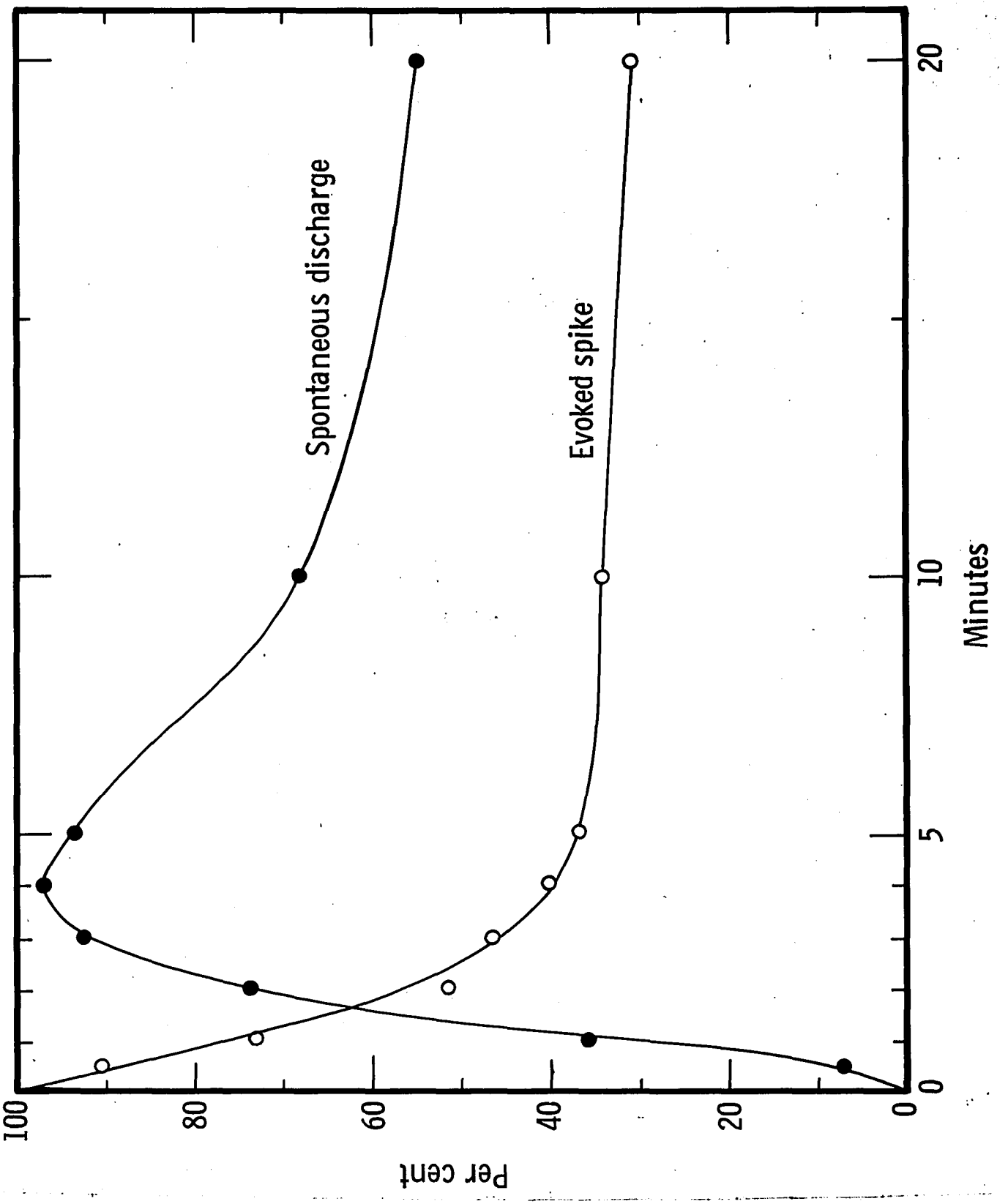


Figure 2. The time course of spontaneous discharge and the changes in amplitude of the evoked alpha-A fiber spike in desheathed nerves soaked in Ca-free Ringer. Each point is an average of 9-11 separate experiments. Amplitudes of spontaneous discharge and evoked spike were calculated as in Fig. 1.

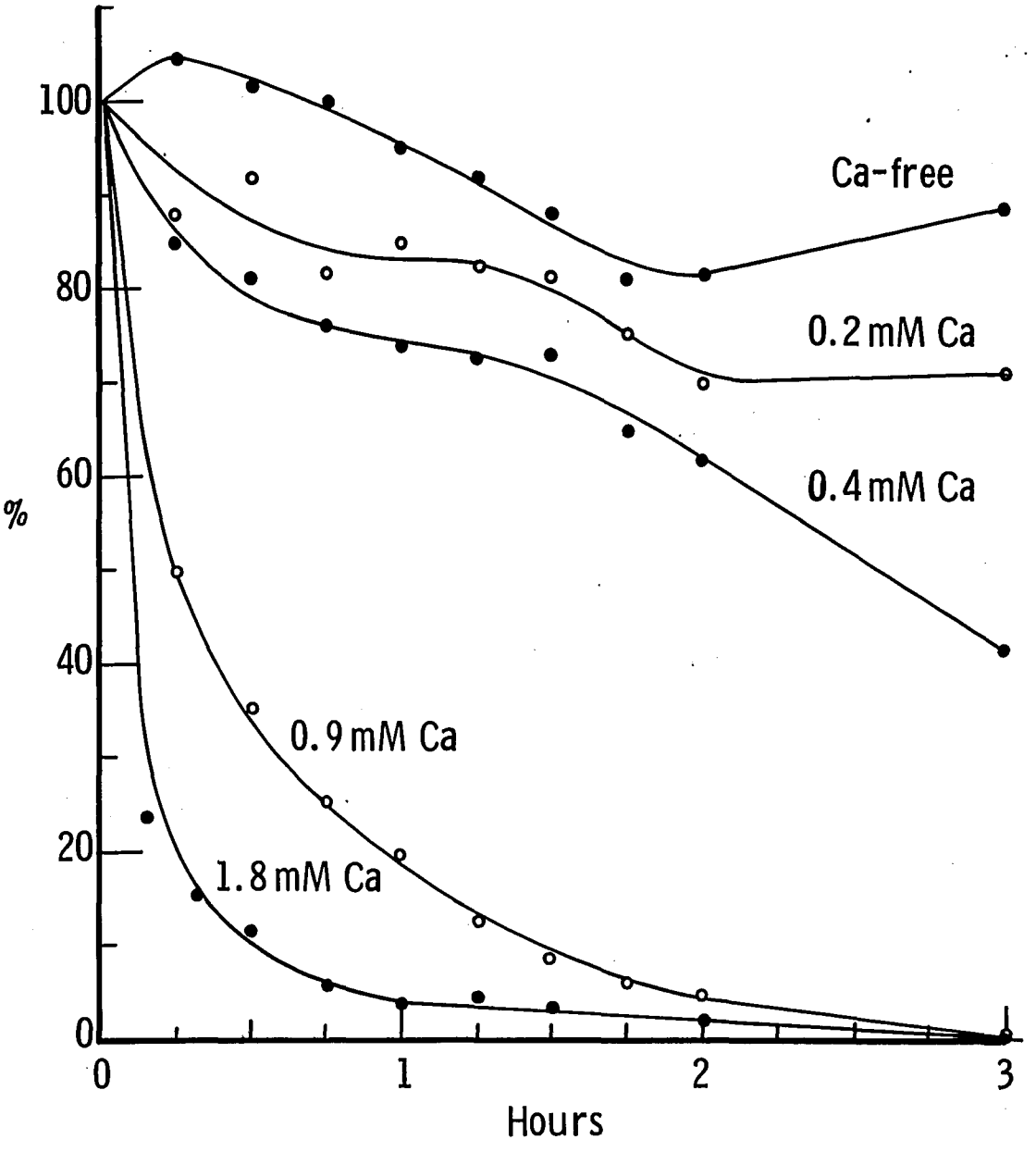


Figure 3. Antagonism of spontaneous discharge by different concentrations of Ca in ensheathed nerves. Each point is an average of 6 separate experiments. Spontaneous discharge amplitudes were calculated as percentages of amplitudes present at time when Ca-free nerve bath was replaced by test solution.

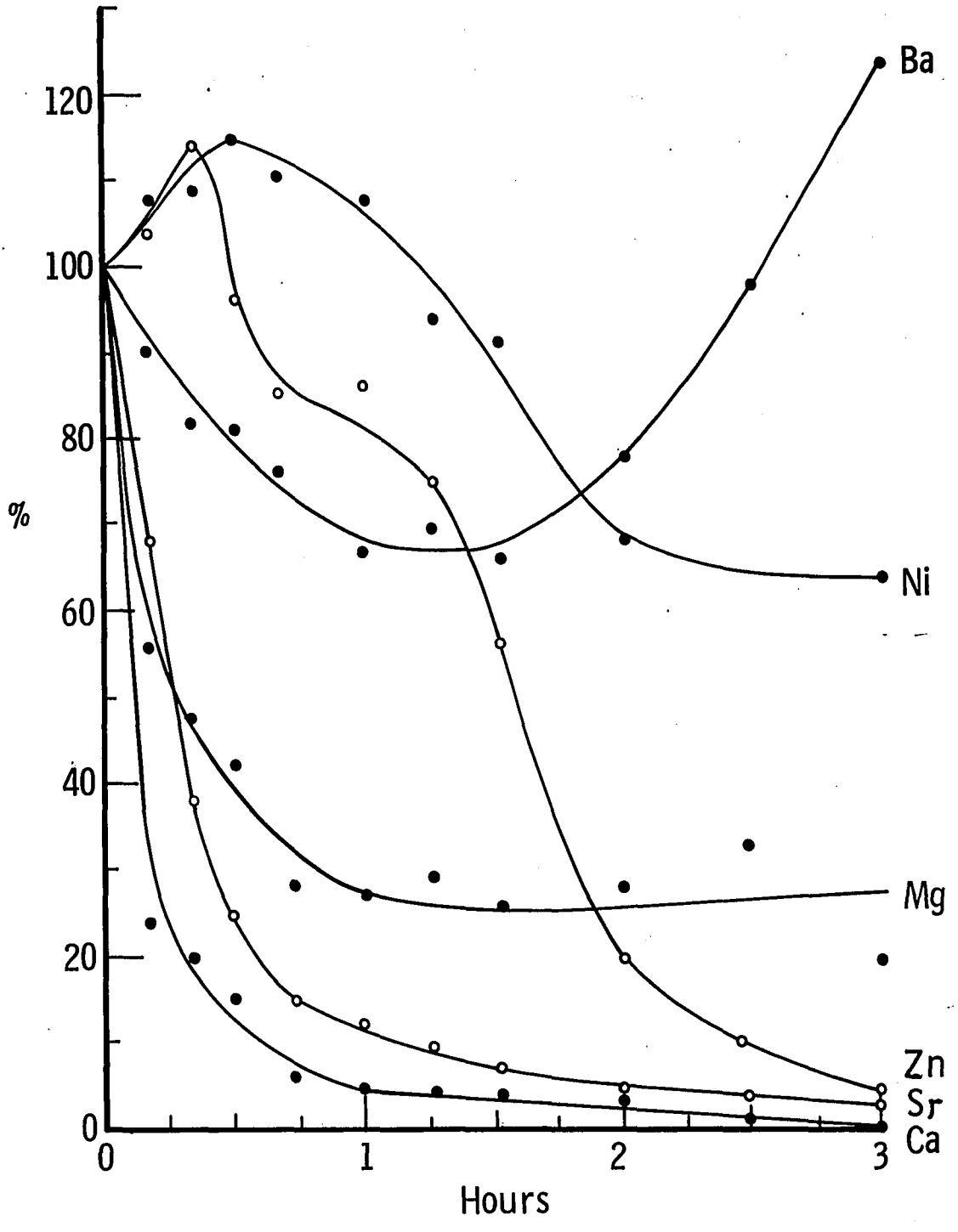


Figure 4. Antagonism of spontaneous discharge by Ca and some other divalent cations in ensheathed nerves. Each point is an average of 6 separate experiments. Spontaneous discharge amplitudes were calculated as in Fig. 3.

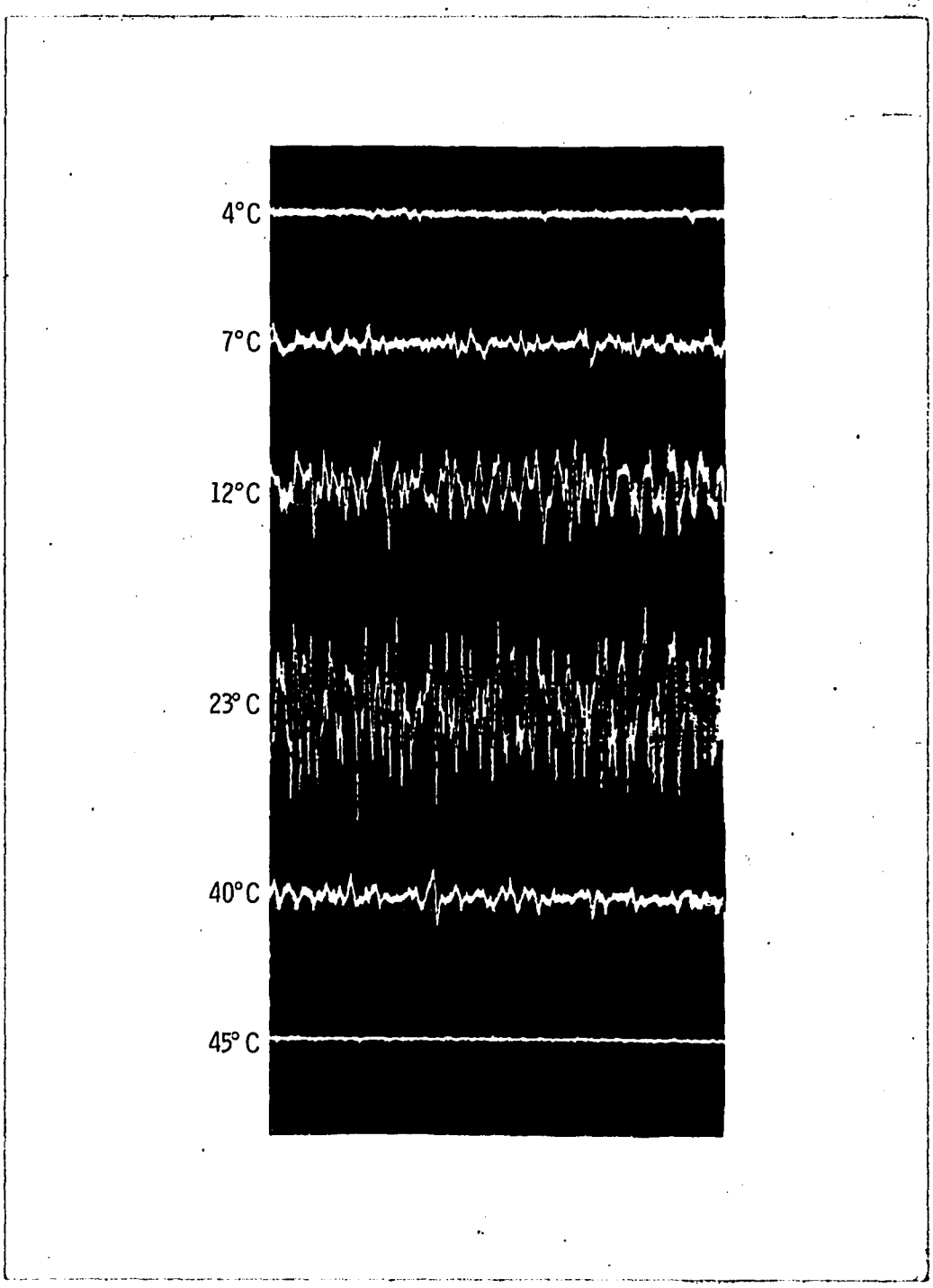


Figure 5. Effects of changes in temperature on spontaneous discharge in ensheathed nerve. Nerve was immersed in Ca-free Ringer for 18 hours at 5°C prior to being tested. Each tracing was obtained at 30 seconds after immersion in the Ca-free bath at the temperature indicated. Calibration: 5 msec. and 100 μV.

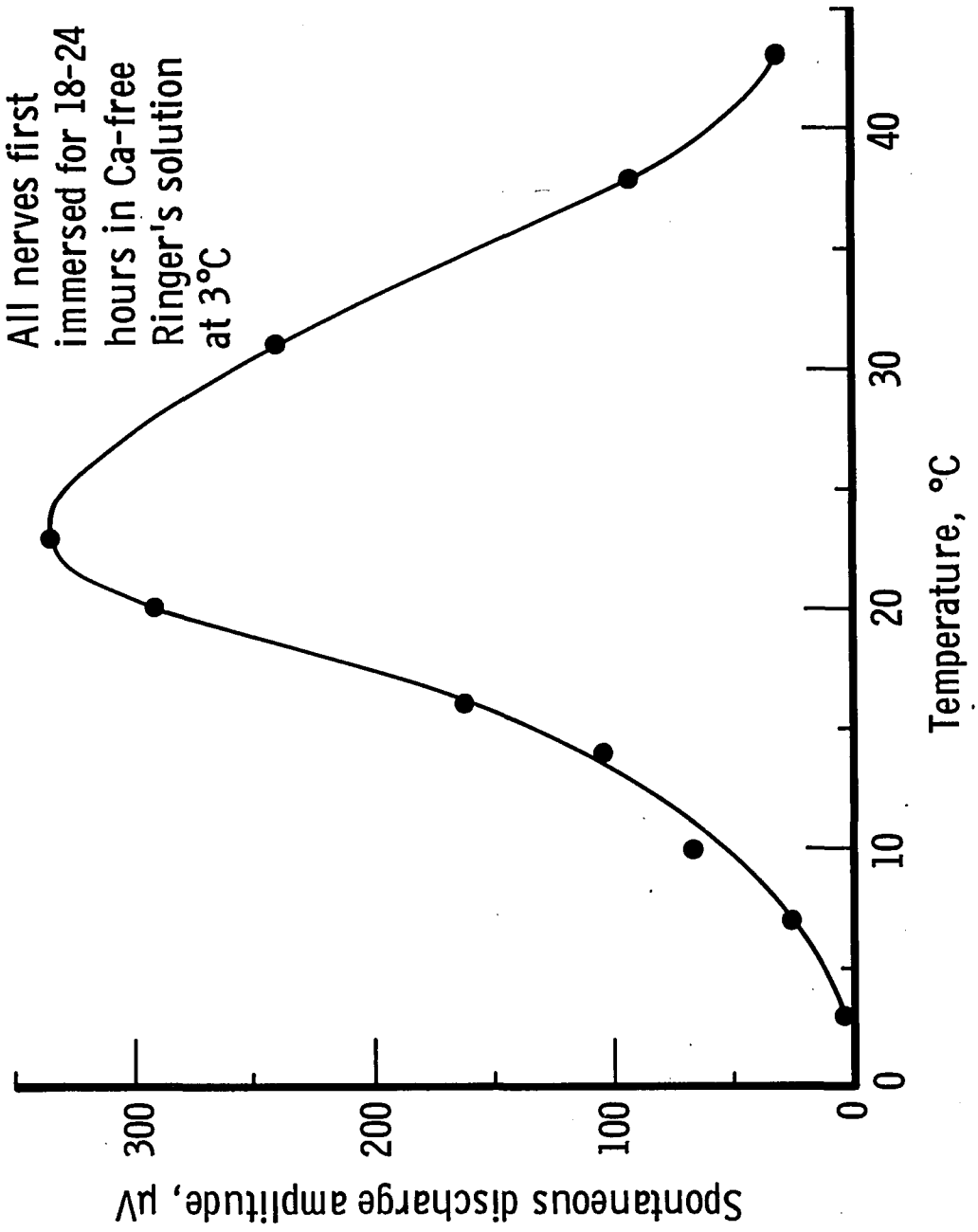


Figure 6. Effects of changes in temperature on spontaneous discharge in ensheathed nerve. Measurements of spontaneous discharge amplitude were made at 30 seconds after immersion in the test solution. Each point is an average of 6-11 separate experiments.

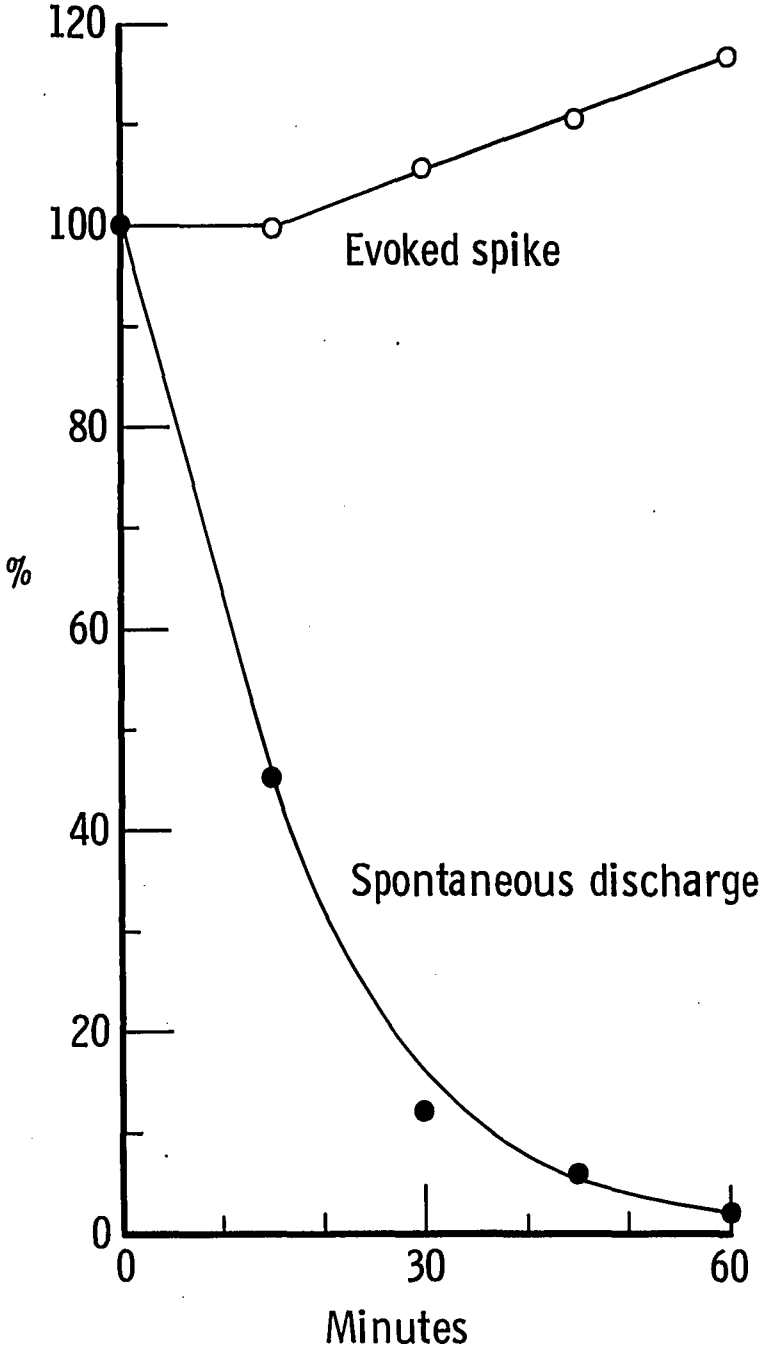


Figure 7. Effect of 10 mM sodium azide on spontaneous discharge and evoked spike in ensheathed nerves. Each point is an average of 6 separate experiments. Nerves were soaked in Ca-free Ringer for 18 hours prior to treatment with sodium azide. Amplitudes of spontaneous discharge and evoked spike were calculated as percentages of amplitudes present at time when Ca-free nerve bath was replaced by azide solution.

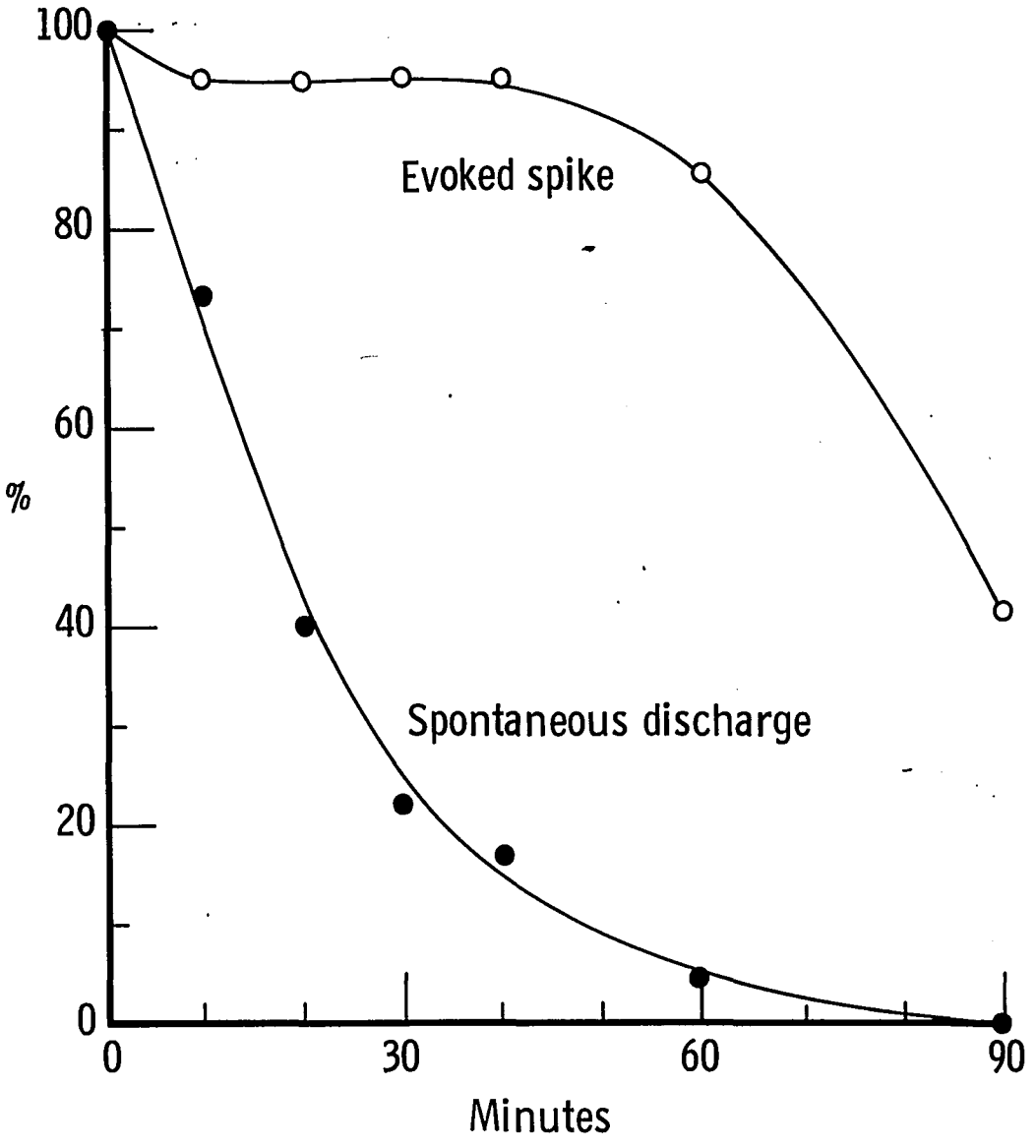


Figure 8. Effect of 0.5 mM DNP on spontaneous discharge and evoked spike in ensheathed nerves. Each point is an average of 6 separate experiments. Experimental procedure was the same as described in Fig. 7.

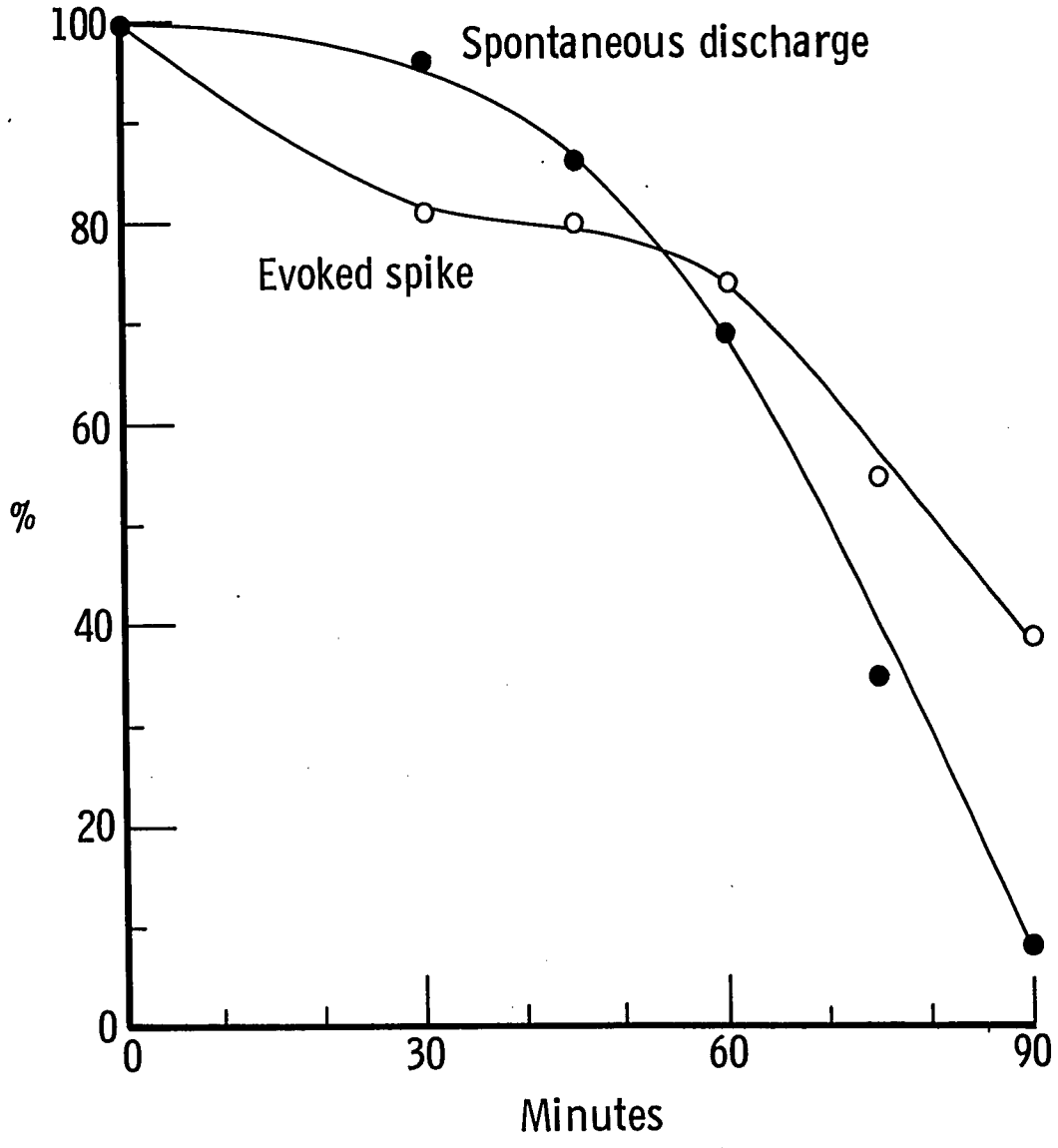


Figure 9. Effect of 10 mM sodium iodoacetate on spontaneous discharge and evoked spike in ensheathed nerves. Each point is an average of 6 separate experiments. Experimental procedure was the same as described in Fig. 7.

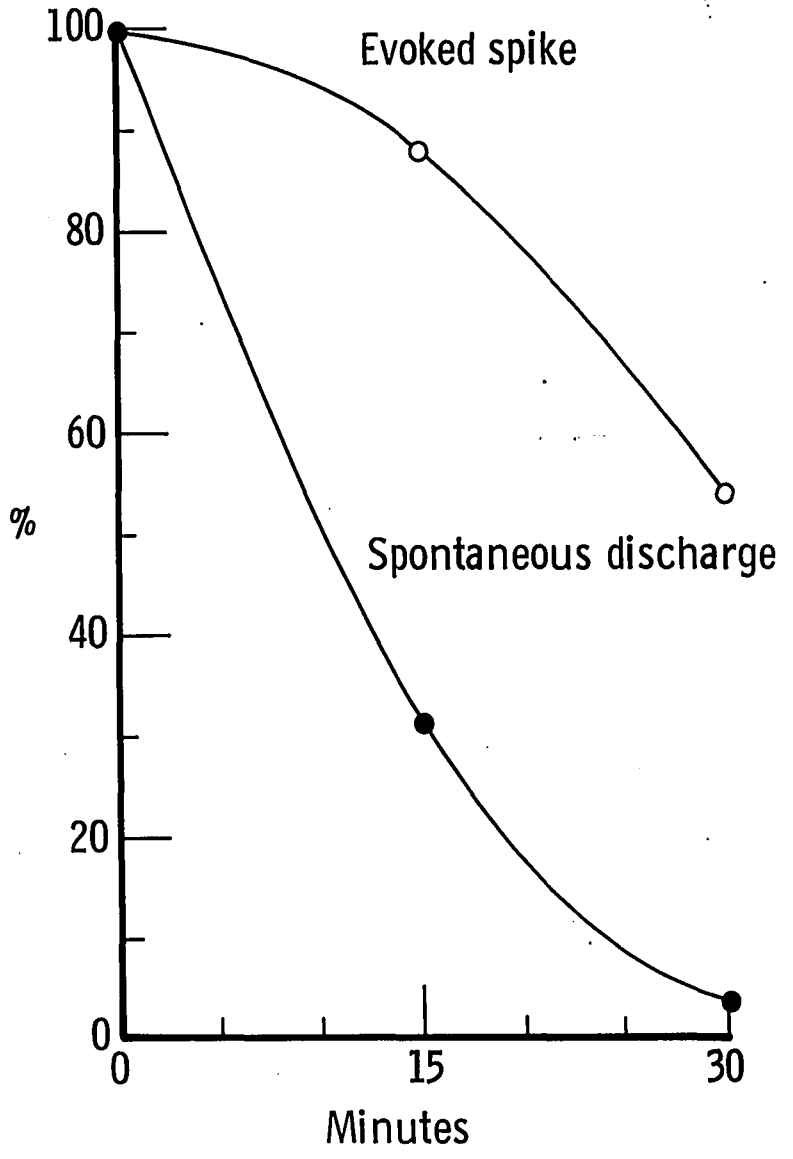


Figure 10. Effect of 0.5 mM N-ethylmaleimide on spontaneous discharge and evoked spike in ensheathed nerves. Each point is an average of 6 separate experiments. Experimental procedure was the same as described in Fig. 7.

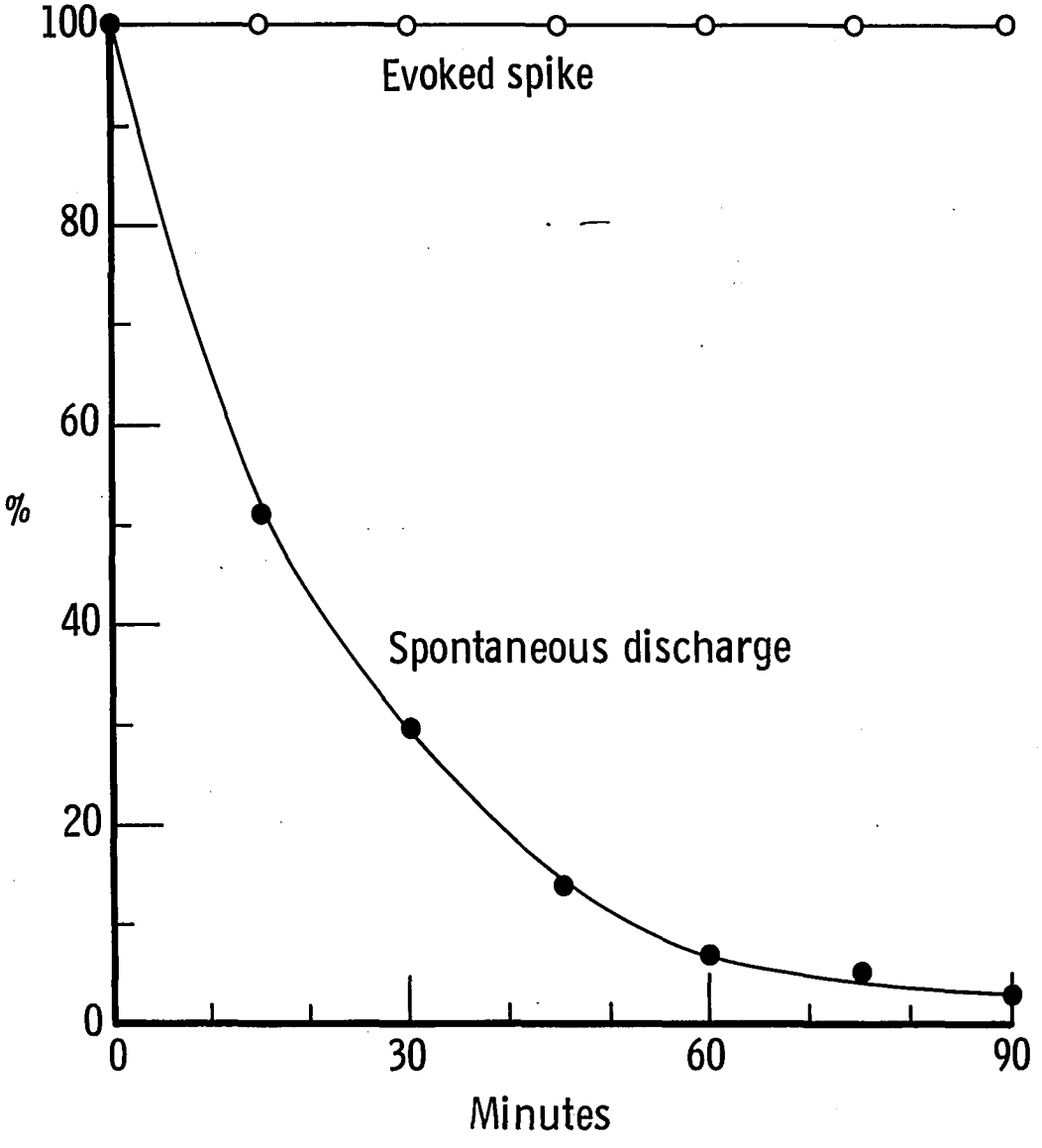


Figure 11. Effect of 1 mM physostigmine on spontaneous discharge and evoked spike in ensheathed nerves. Each point is an average of 6 separate experiments. Experimental procedure was the same as described in Fig. 7.

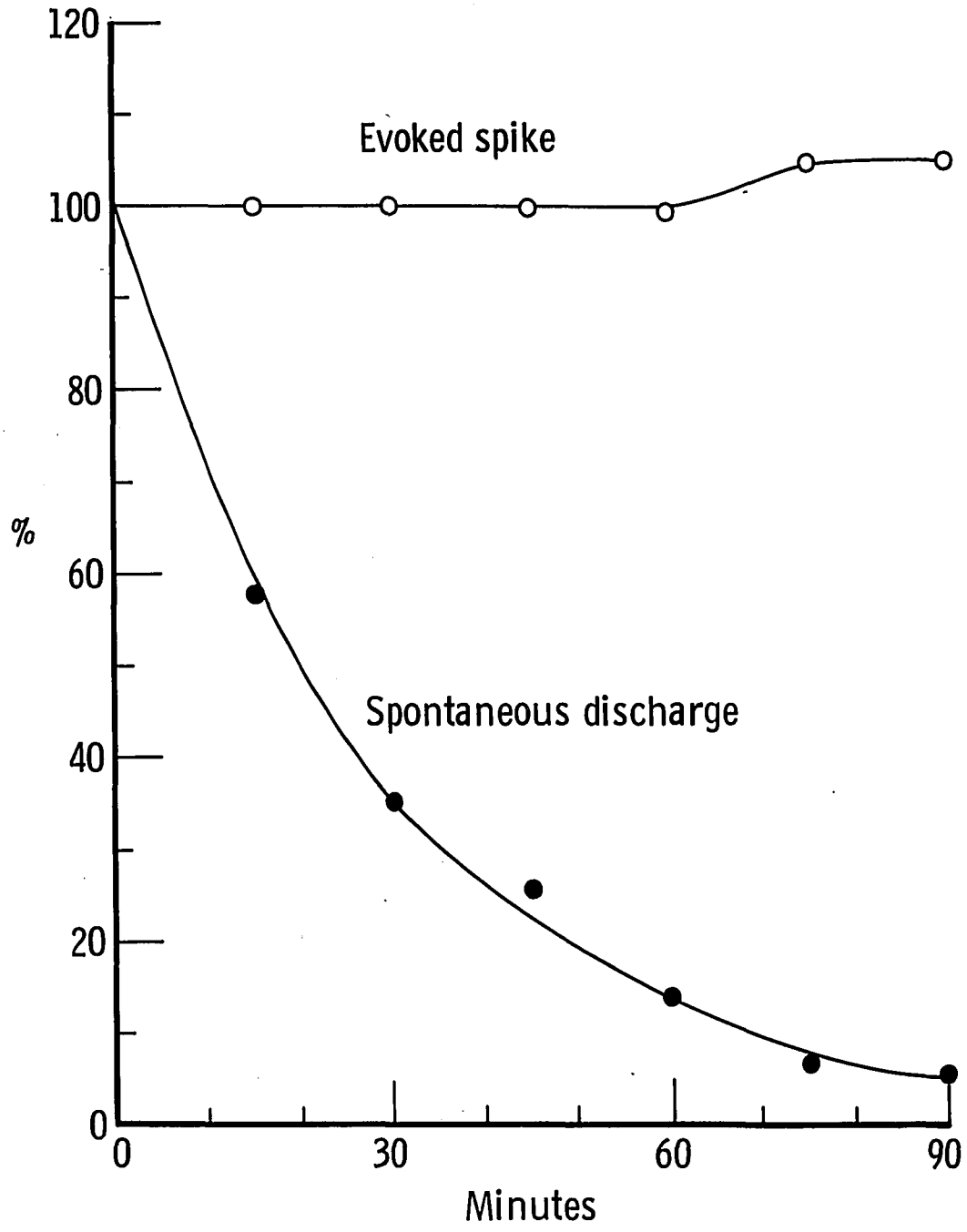


Figure 12. Effect of 5 mM caffeine on spontaneous discharge and evoked spike in ensheathed nerves. Each point is an average of 6 separate experiments. Experimental procedure was the same as described in Fig. 7.

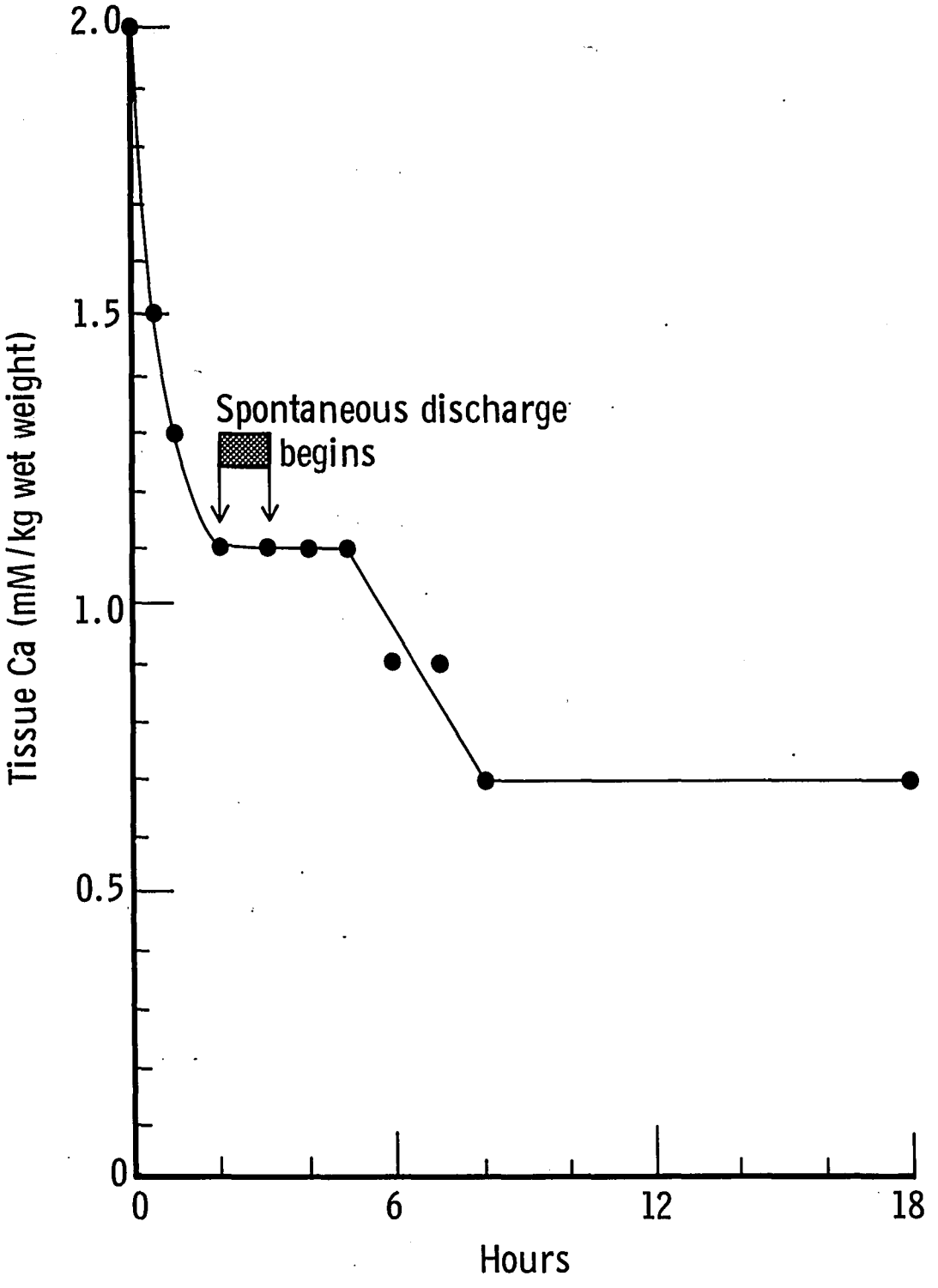


Figure 13. Effect of Ca-free Ringer solution on Ca content of ensheathed nerve. Groups of nerve (4-8) were soaked in the Ca-free Ringer for periods of time indicated on the abscissa, and the Ca content of the entire group was then determined. Each point is the mean of the values obtained from 5-9 separate nerve groups. Ca content shown at zero time is the normal value obtained from freshly dissected nerves or from nerves soaking for 4-5 hours in normal Ringer's.

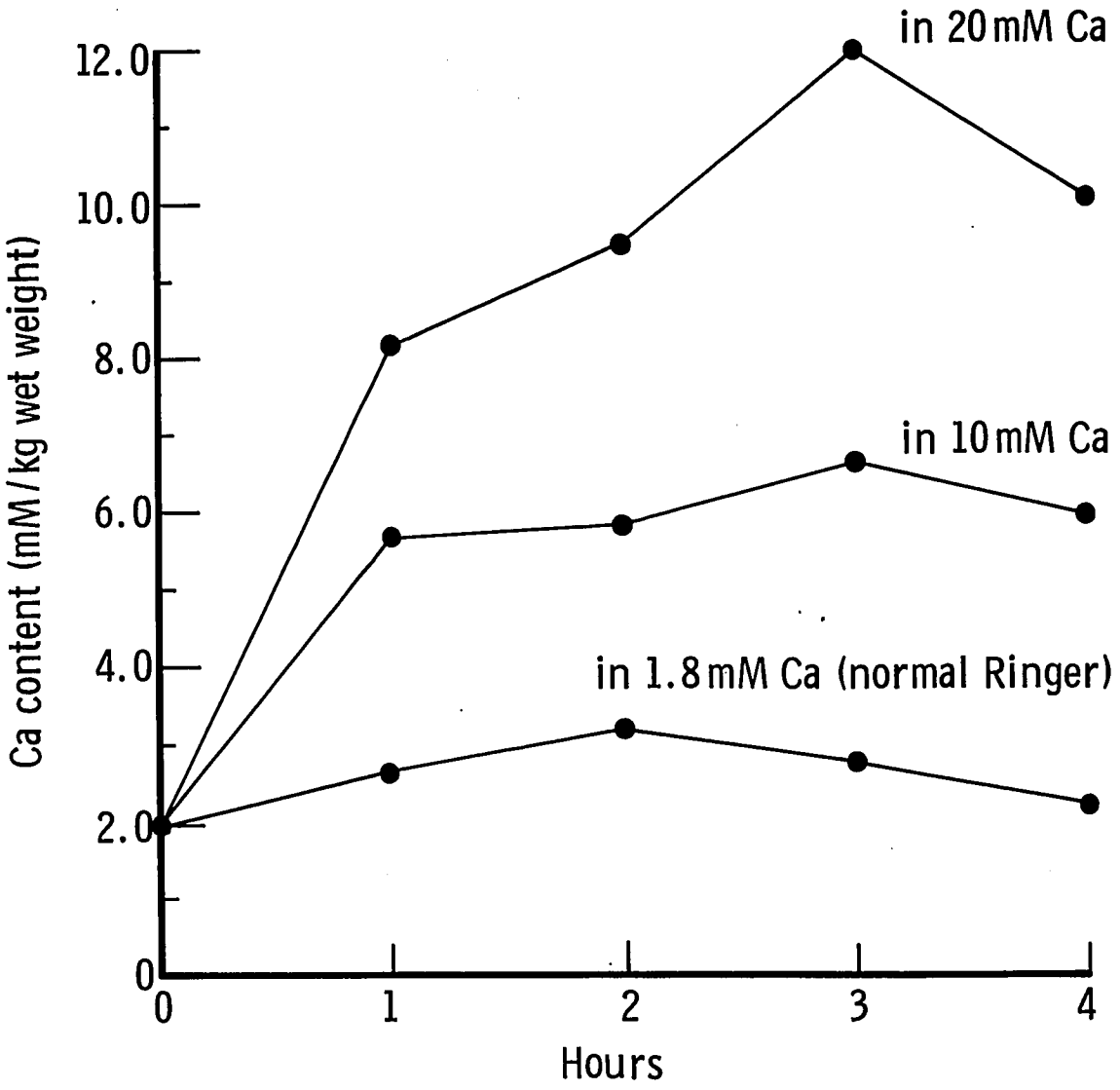


Figure 14. Effects of normal and high-Ca Ringer solution on the Ca content of ensheathed nerve. See Fig. 13 for procedure. Each point is the mean of the values obtained from 6 separate nerve groups (4 nerves in each group).

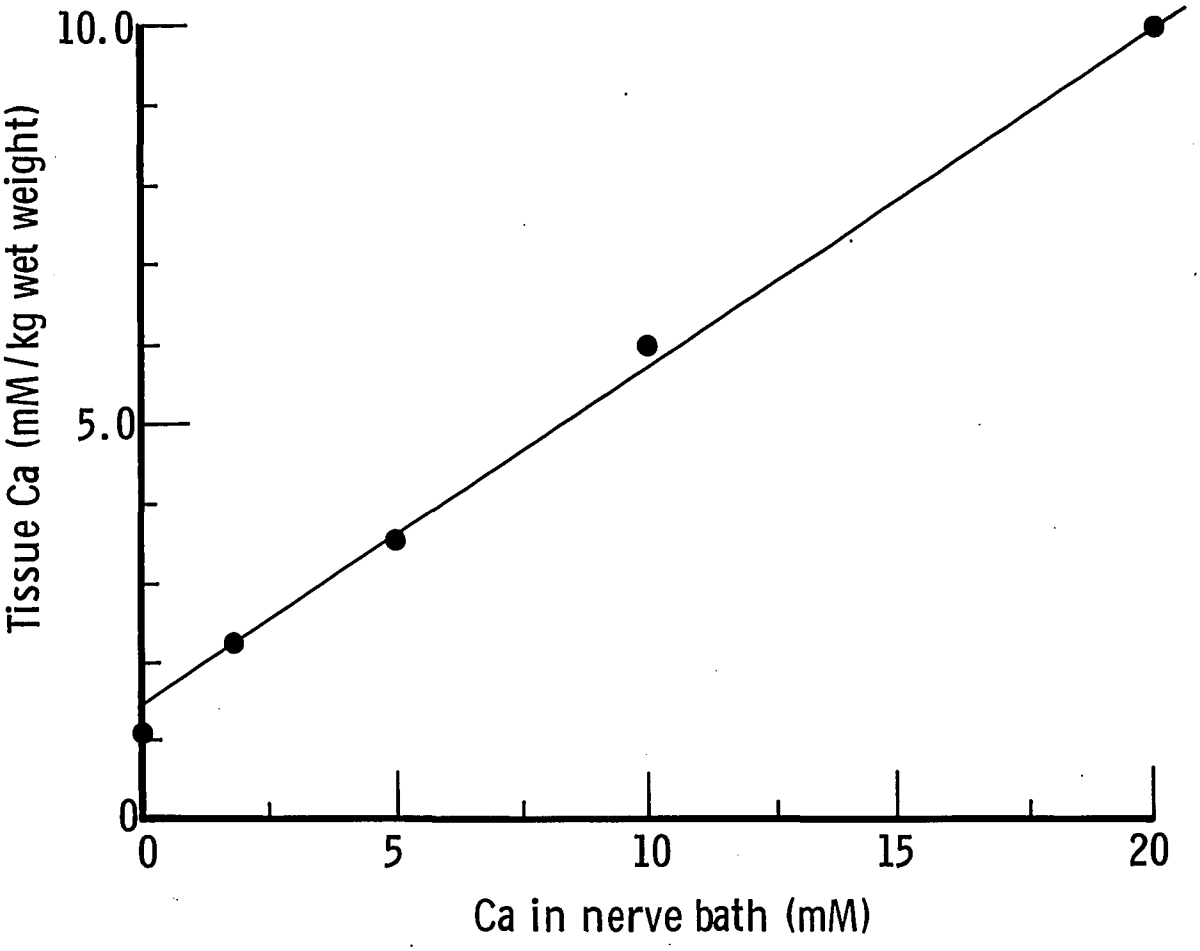


Figure 15. Relationship between Ca concentration in bath and Ca content in ensheathed nerve. Groups of nerves were soaked in Ringer solutions containing 0 to 20 mM Ca for exactly 4 hours. Each point is the mean of 6 separate groups.

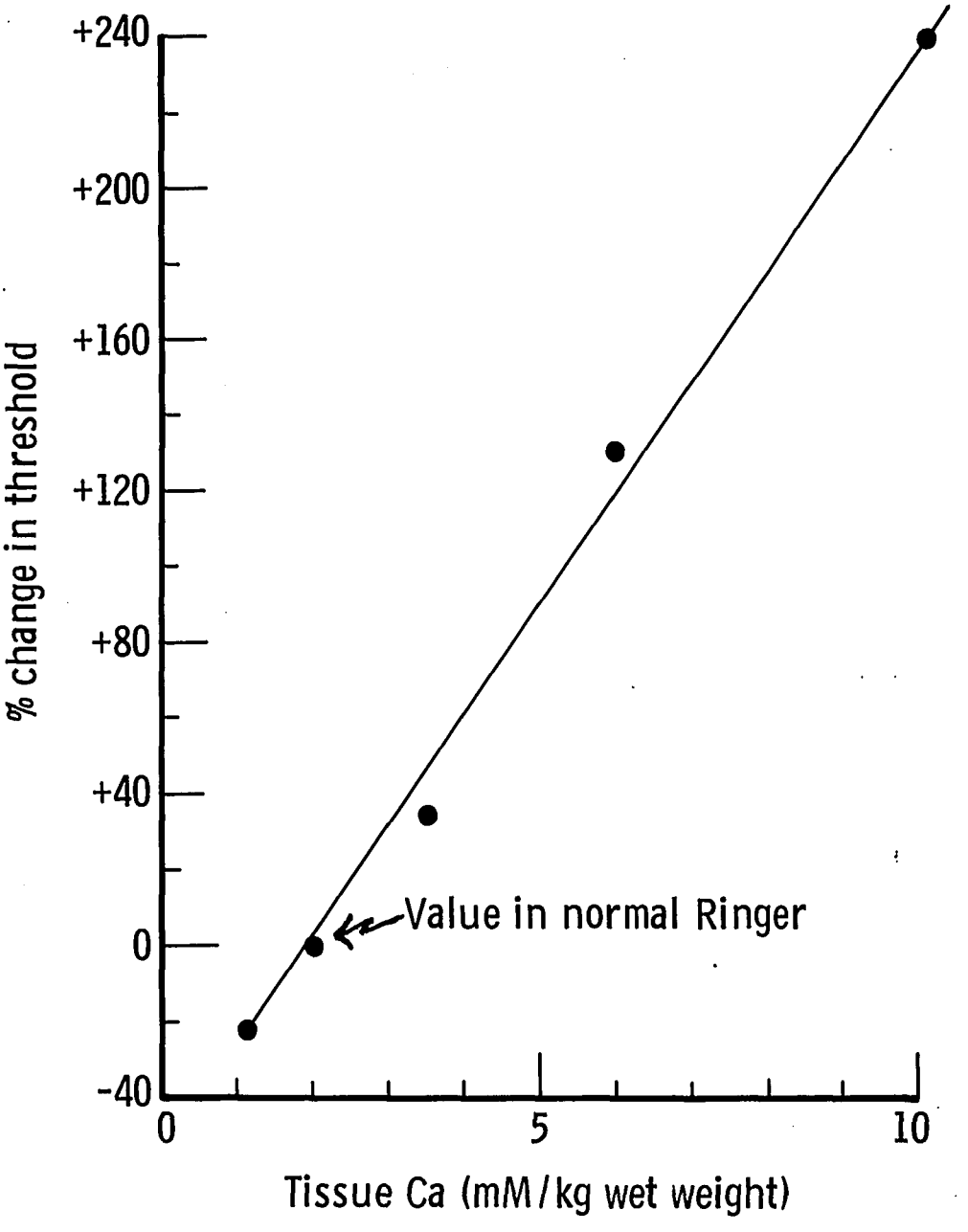


Figure 16. Relationship between Ca content of ensheathed nerve and excitability. Zero value on ordinate represents excitability in normal Ringer's. Nerves with different Ca contents obtained by soaking nerves for 4 hours in Ringer solutions containing 0 to 20 mM Ca.

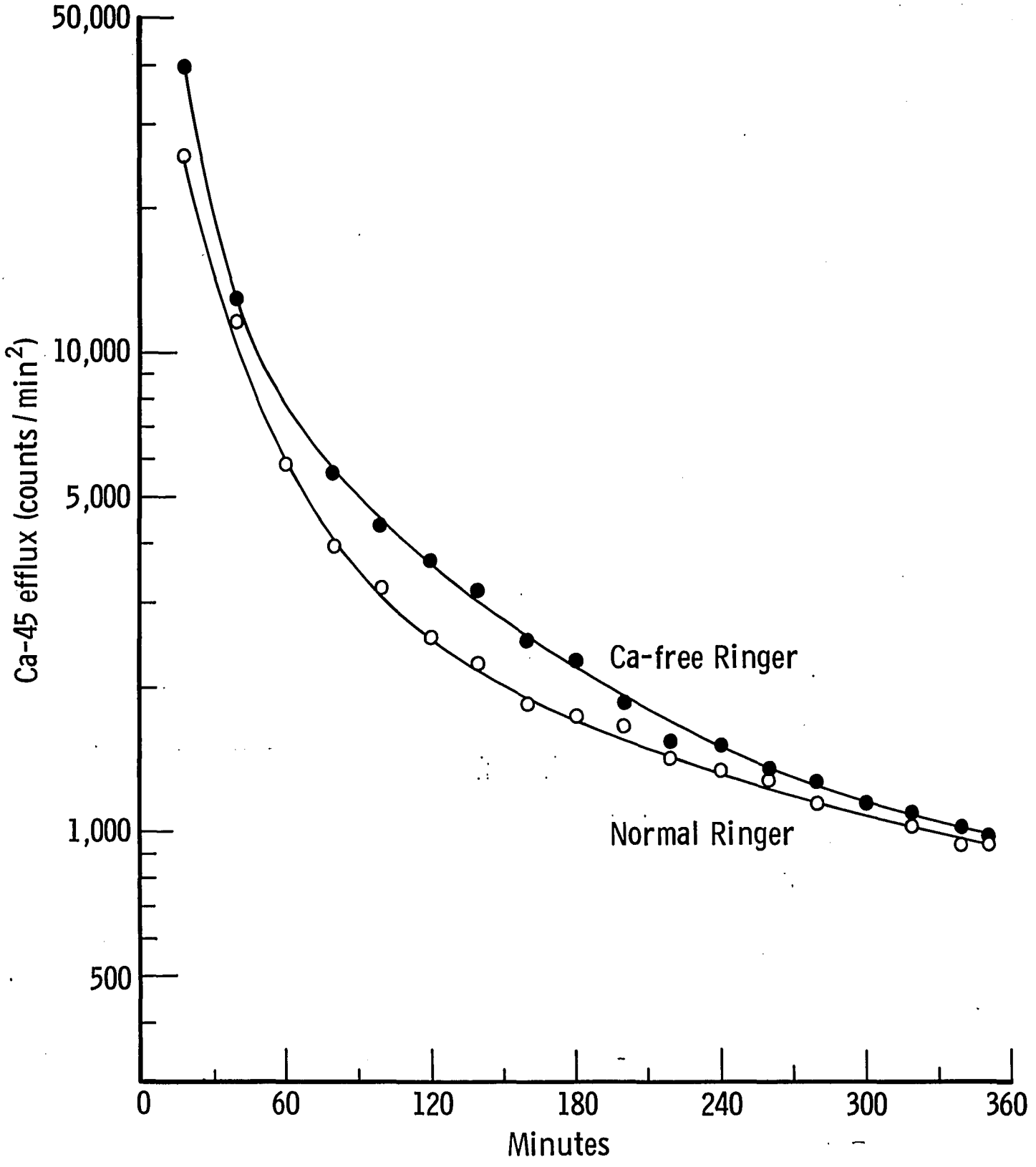


Figure 17. The time course of the rate of Ca-45 efflux into normal or Ca-free Ringer's from ensheathed nerves previously soaked in normal Ringer's containing Ca-45. Each point is the mean of 6 separate experiments.

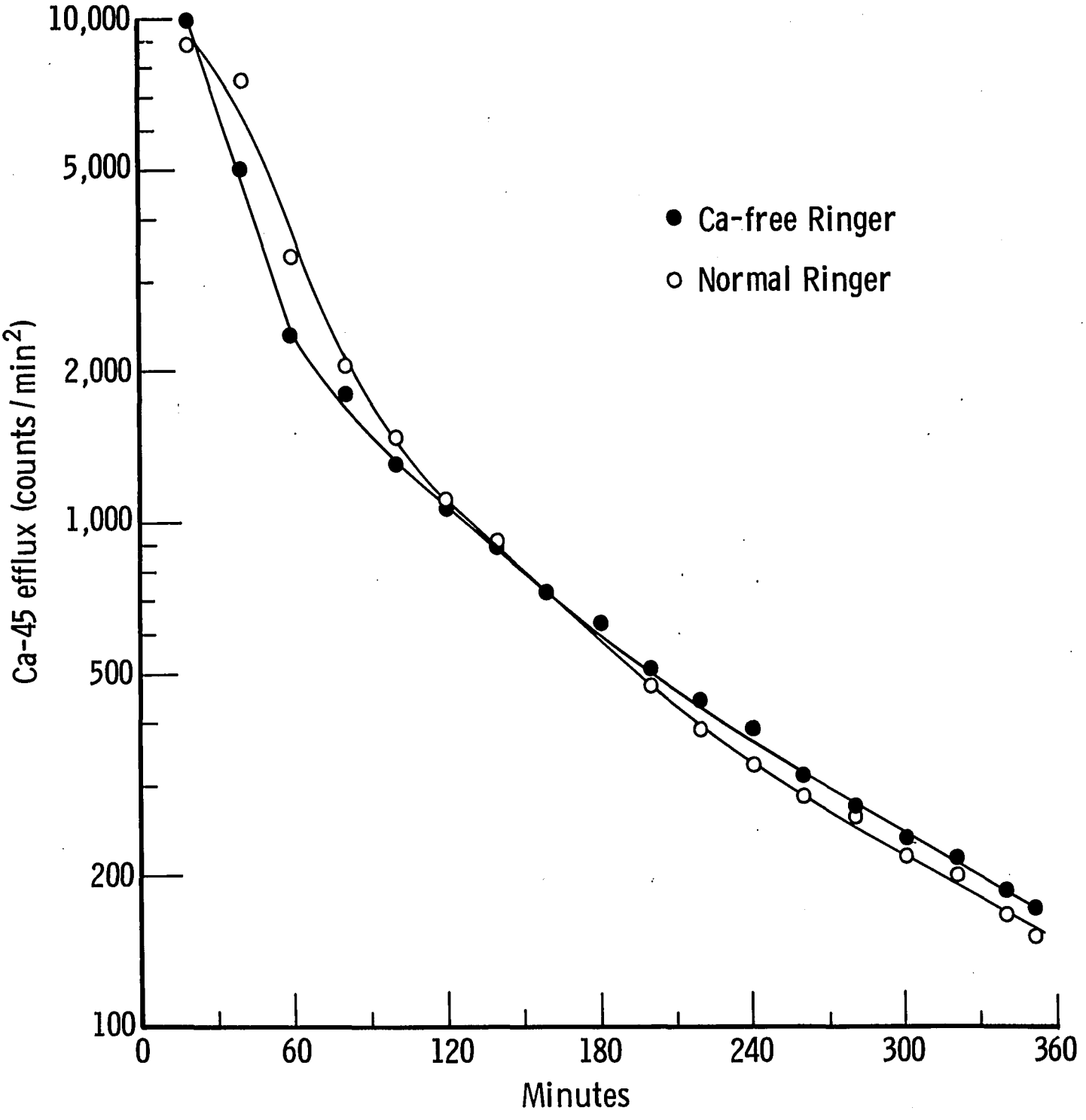


Figure 18. The time course of the rate of Ca-45 efflux into normal or Ca-free Ringer's from desheathed nerves previously soaked in normal Ringer's containing Ca-45. Each point is the mean of 6 separate experiments.

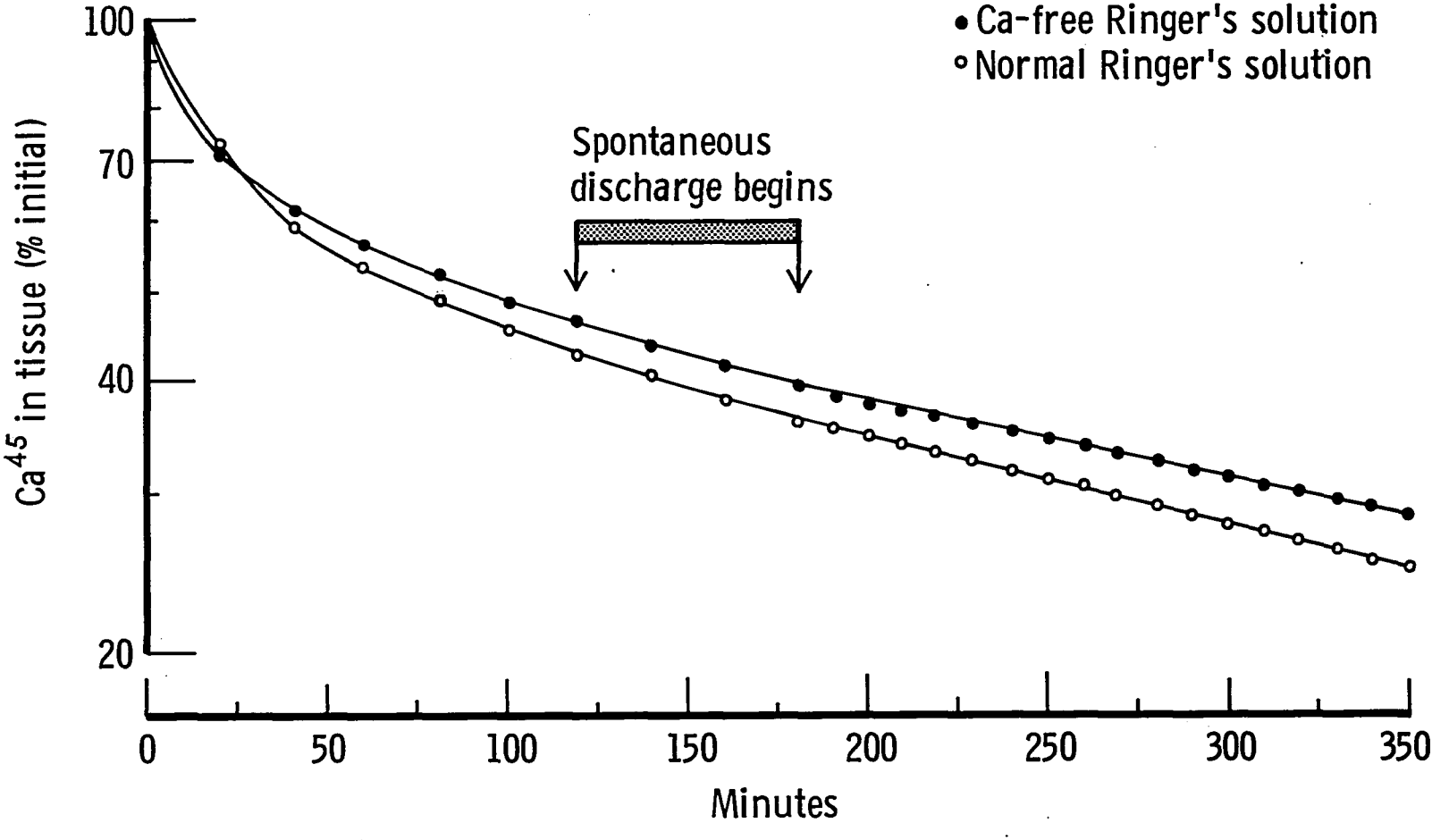


Figure 19. The time course of decline of Ca-45 in ensheathed nerves soaking in normal of Ca-free Ringer's. The range of times of appearance of spontaneous discharge in Ca-deficient ensheathed nerves is noted on the graph. Data obtained from same noted on the graph. Experiments as in Fig. 17.

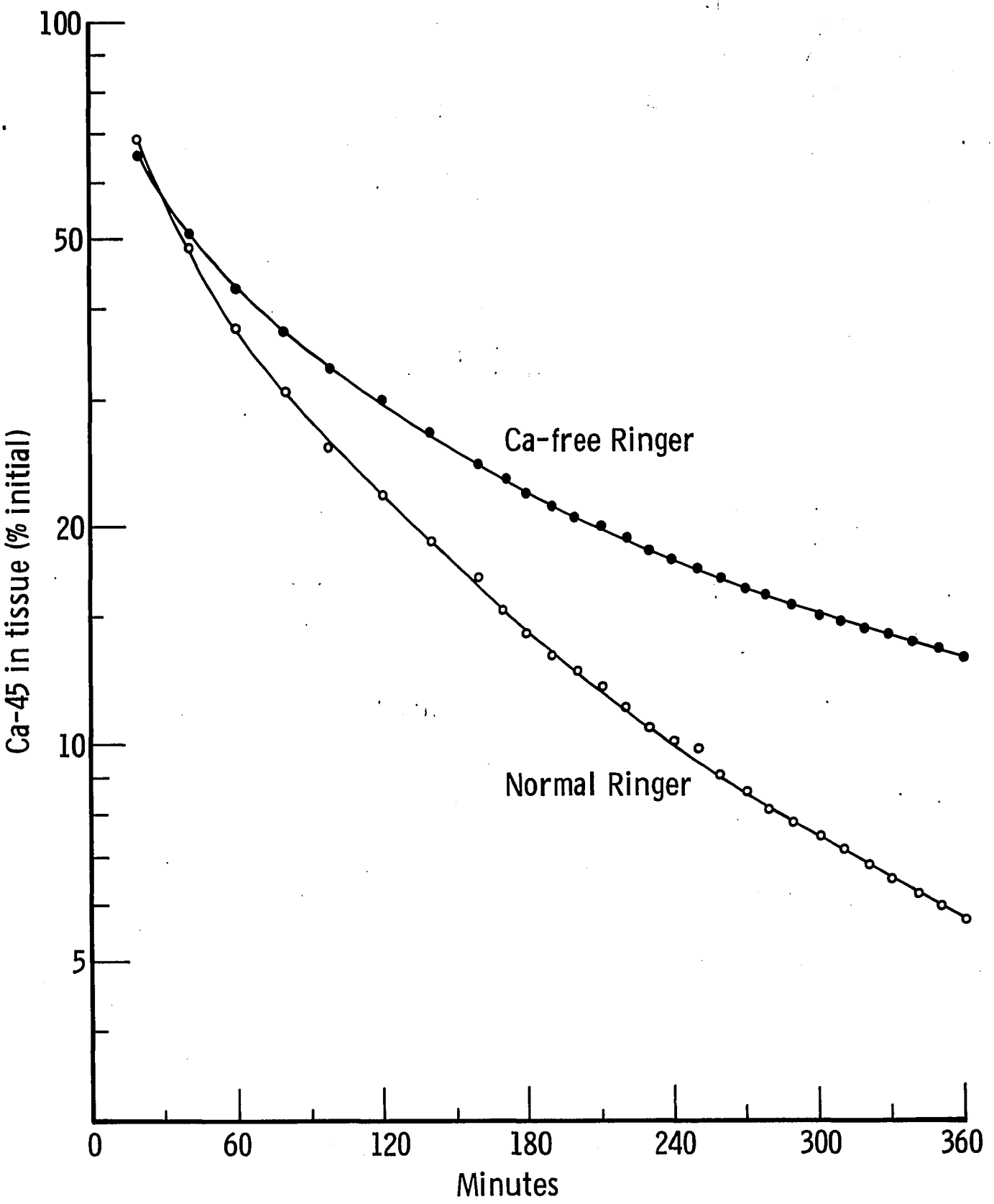


Figure 20. The time course of decline of Ca-45 in desheathed nerves in normal or Ca-free Ringer's. Data obtained from same experiments as in Fig. 18.

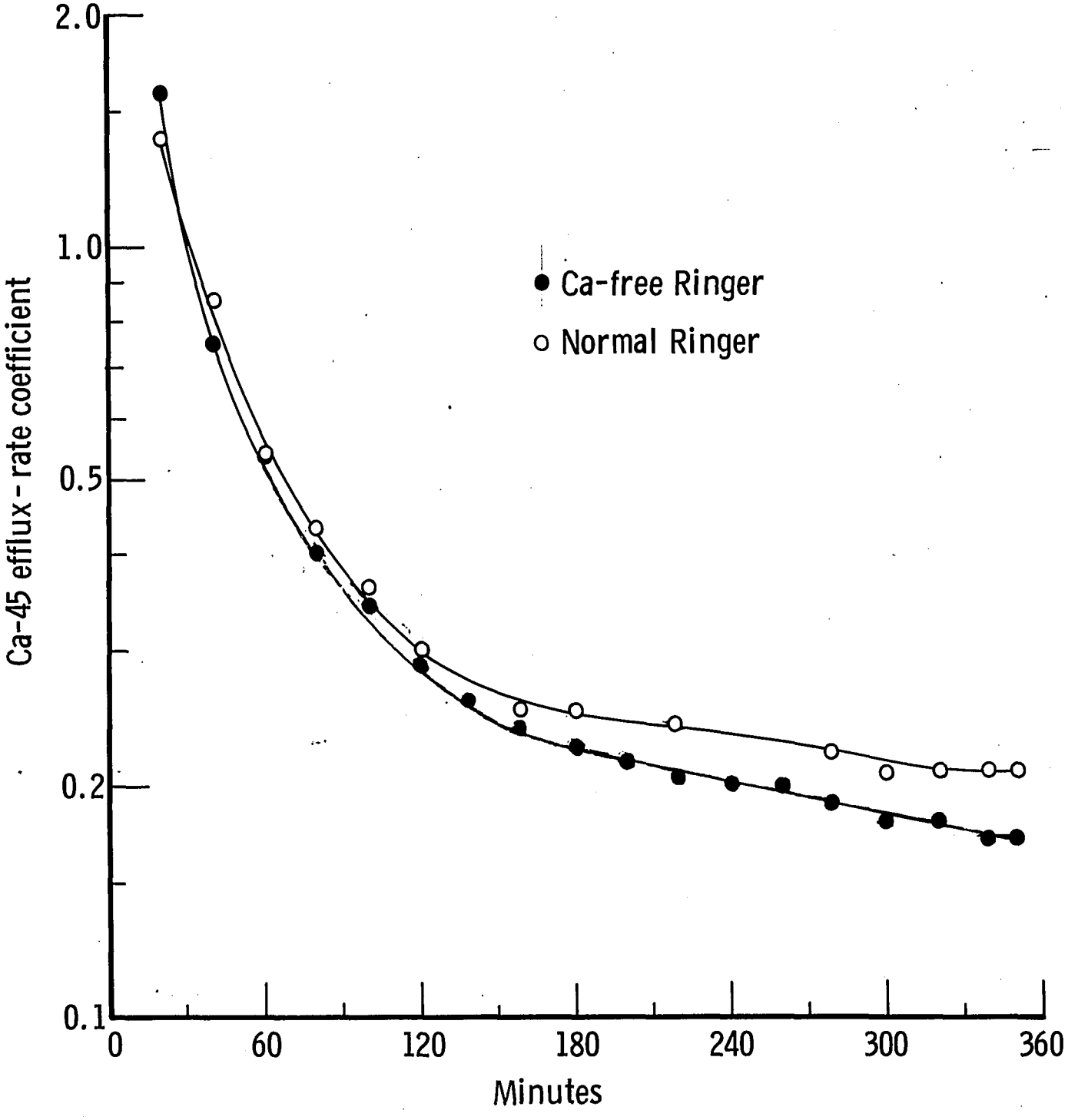


Figure 21. Changes in rate coefficients (%/min) for Ca-45 release from ensheathed nerves into normal or Ca-free Ringer's. Data obtained from same experiments as in Fig. 17.

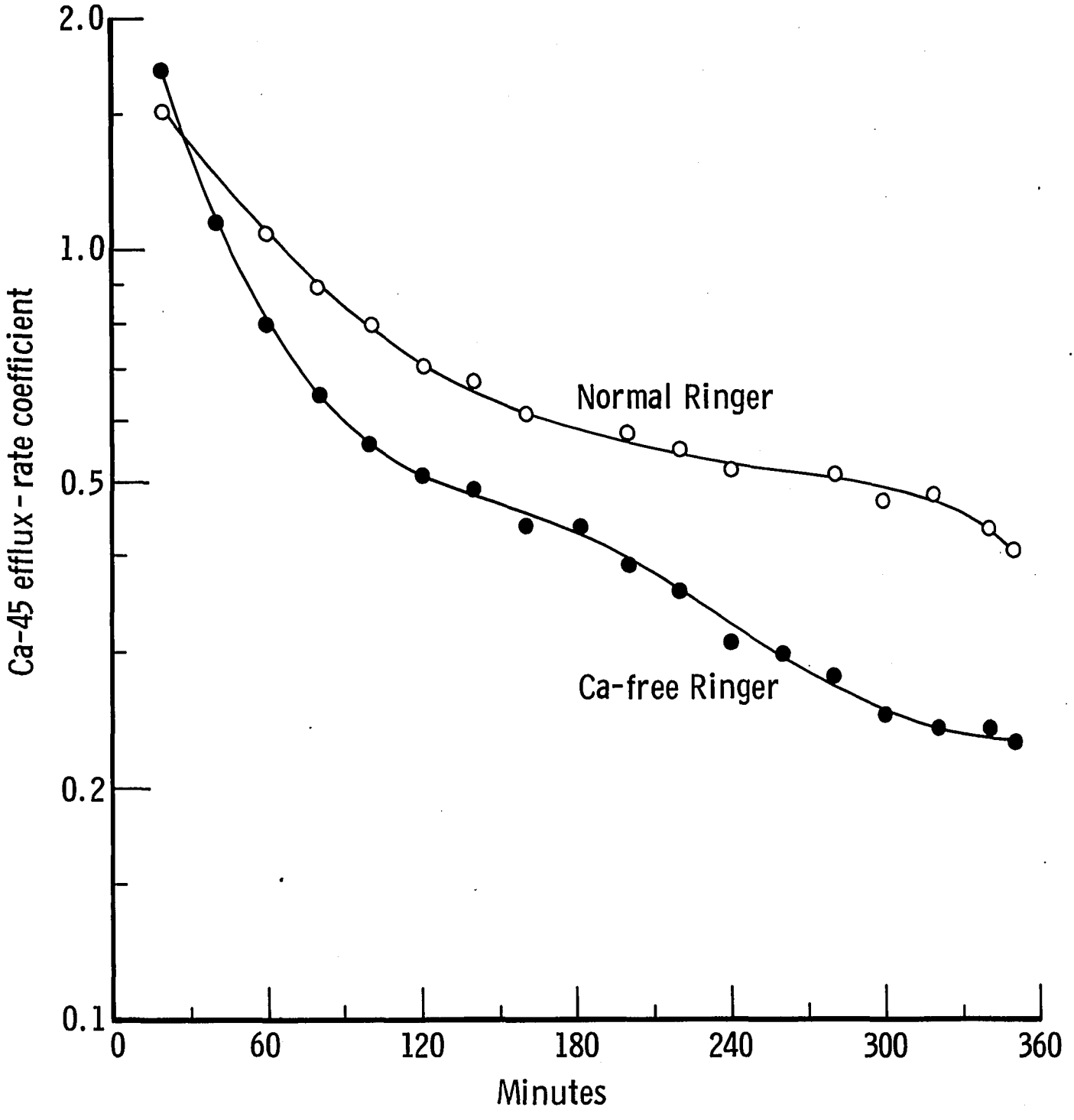


Figure 22. Changes in rate coefficients (%/min) for Ca-45 release from desheathed nerves into normal or Ca-free Ringer's. Data obtained from same experiments as in Fig. 18.

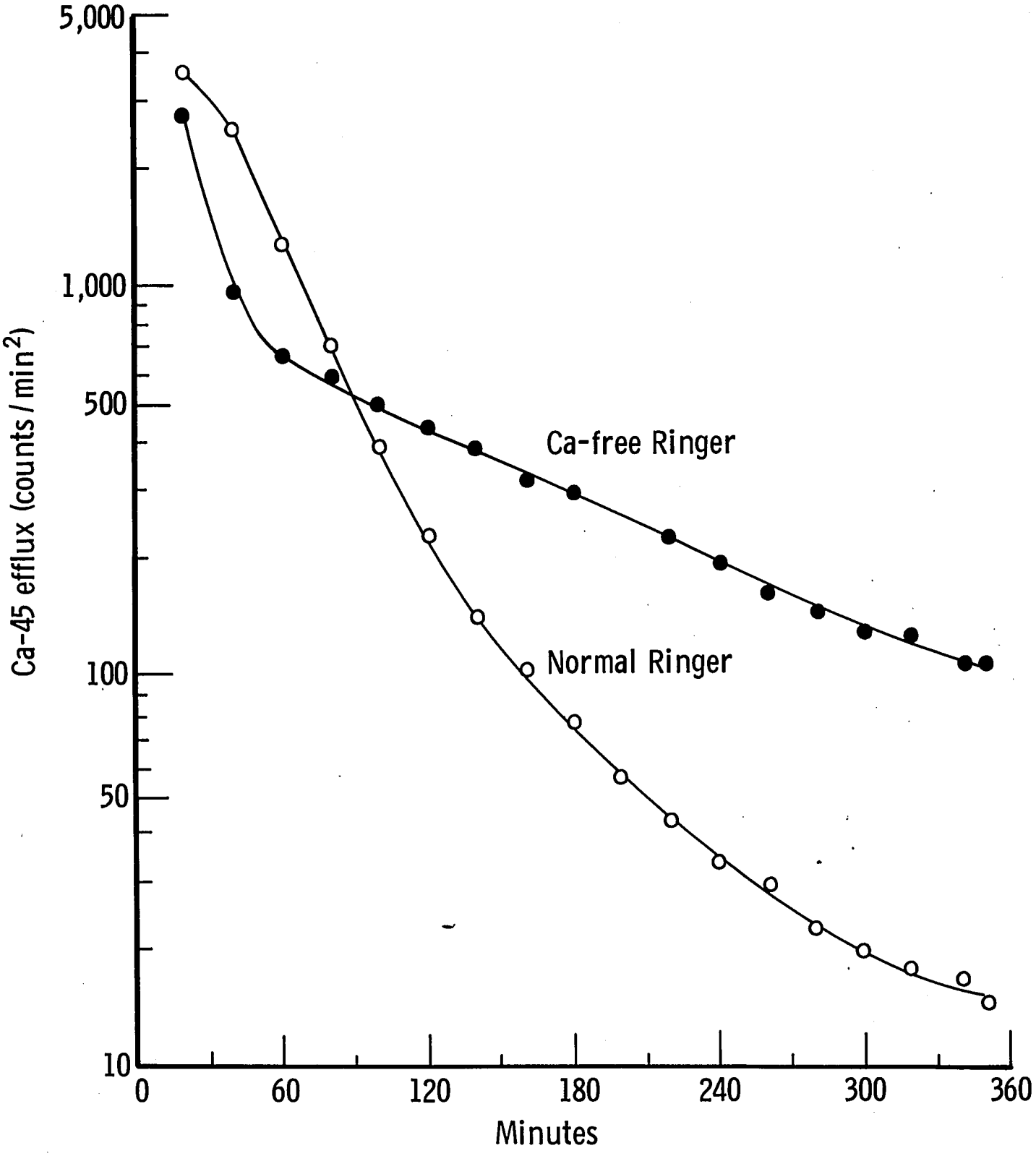


Figure 23. The time course of the rate of Ca-45 release from isolated sheaths into normal or Ca-free Ringer's. Each washout experiment was carried out with 3-4 sheaths, and each point is the mean of 6 separate experiments.

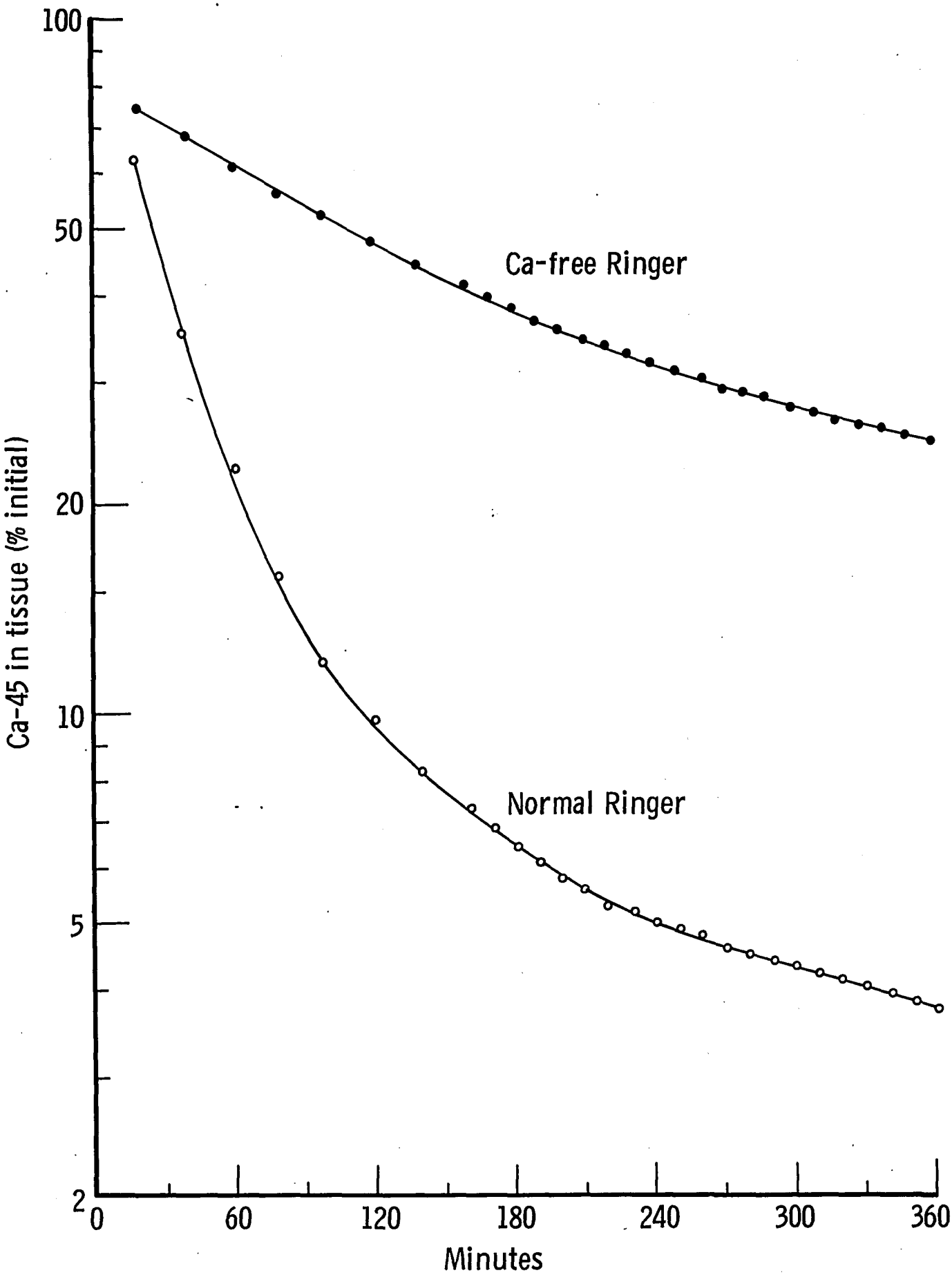


Figure 24. The time course of decline of Ca-45 from isolated sheaths into normal or Ca-free Ringer's. Data obtained from same experiments as in Fig. 23

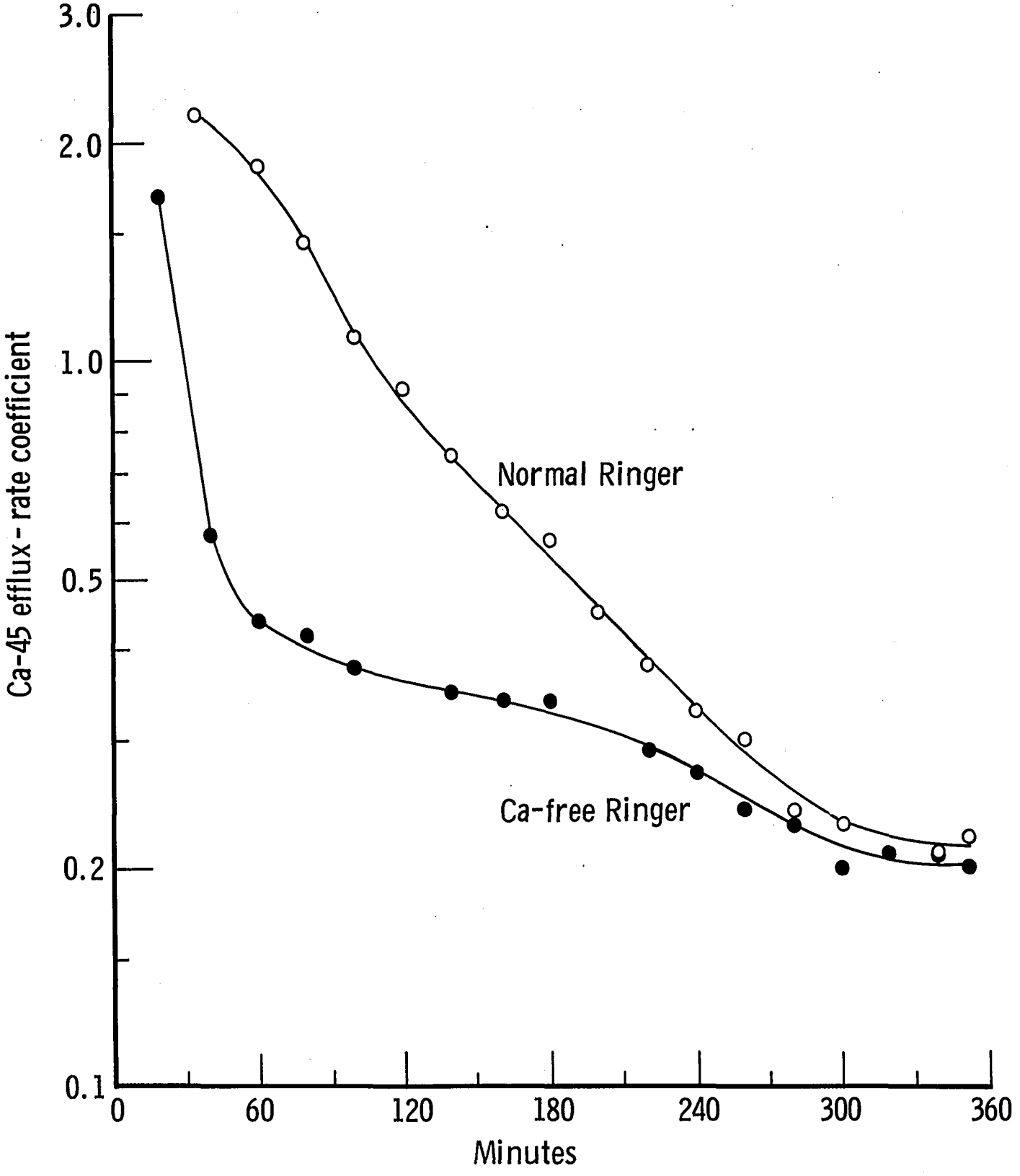


Figure 25. Changes in rate coefficients (%/min) for Ca-45 release from isolated sheaths into normal or Ca-free Ringer's. Data obtained from same experiments as in Fig. 23.

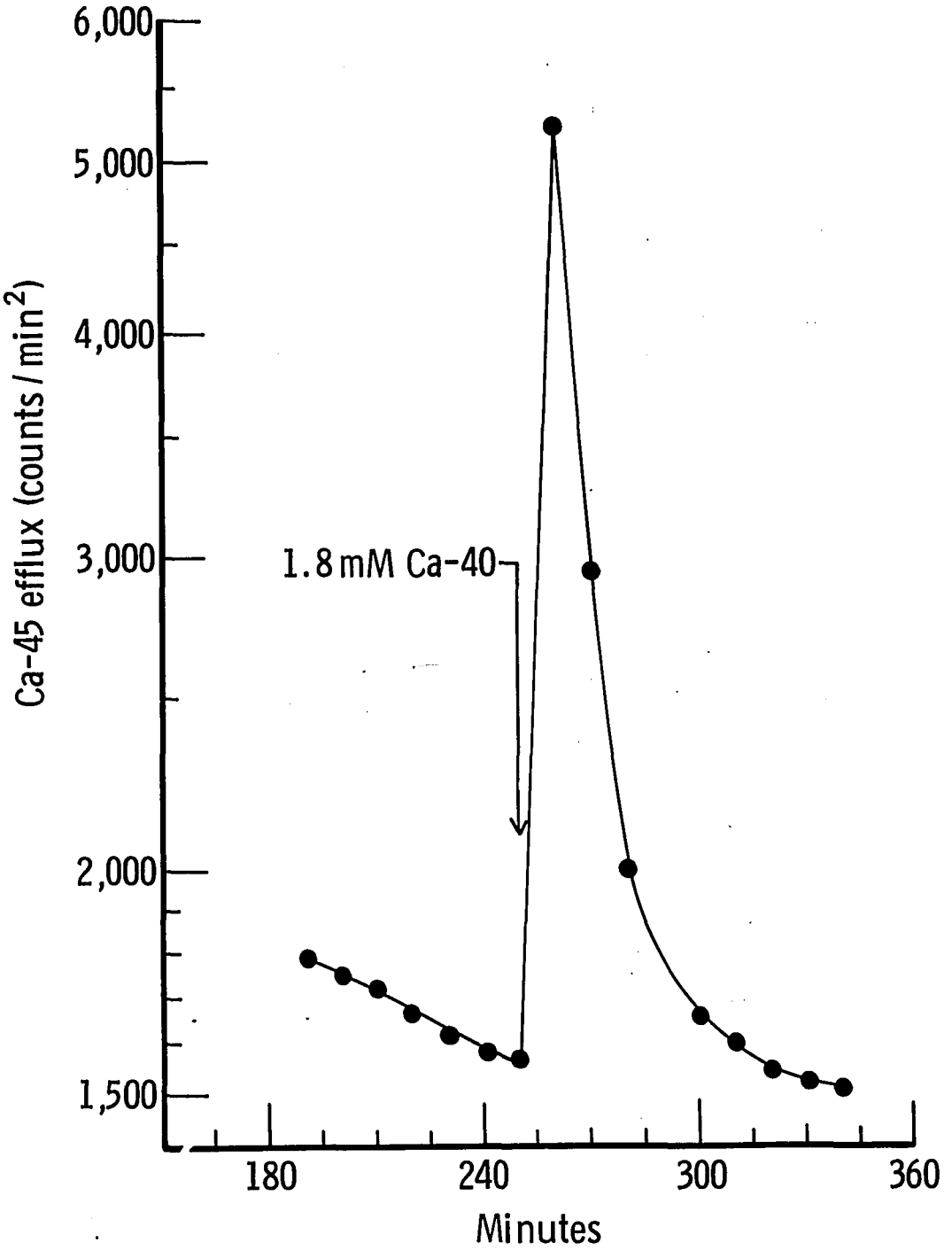


Figure 26. Release of Ca-45 from ensheathed nerve upon addition of 1.8 mM Ca to the nerve bath. Nerves were soaking in Ca-free Ringer's up to the time of addition of Ca. Each point is the mean of 6 separate experiments.

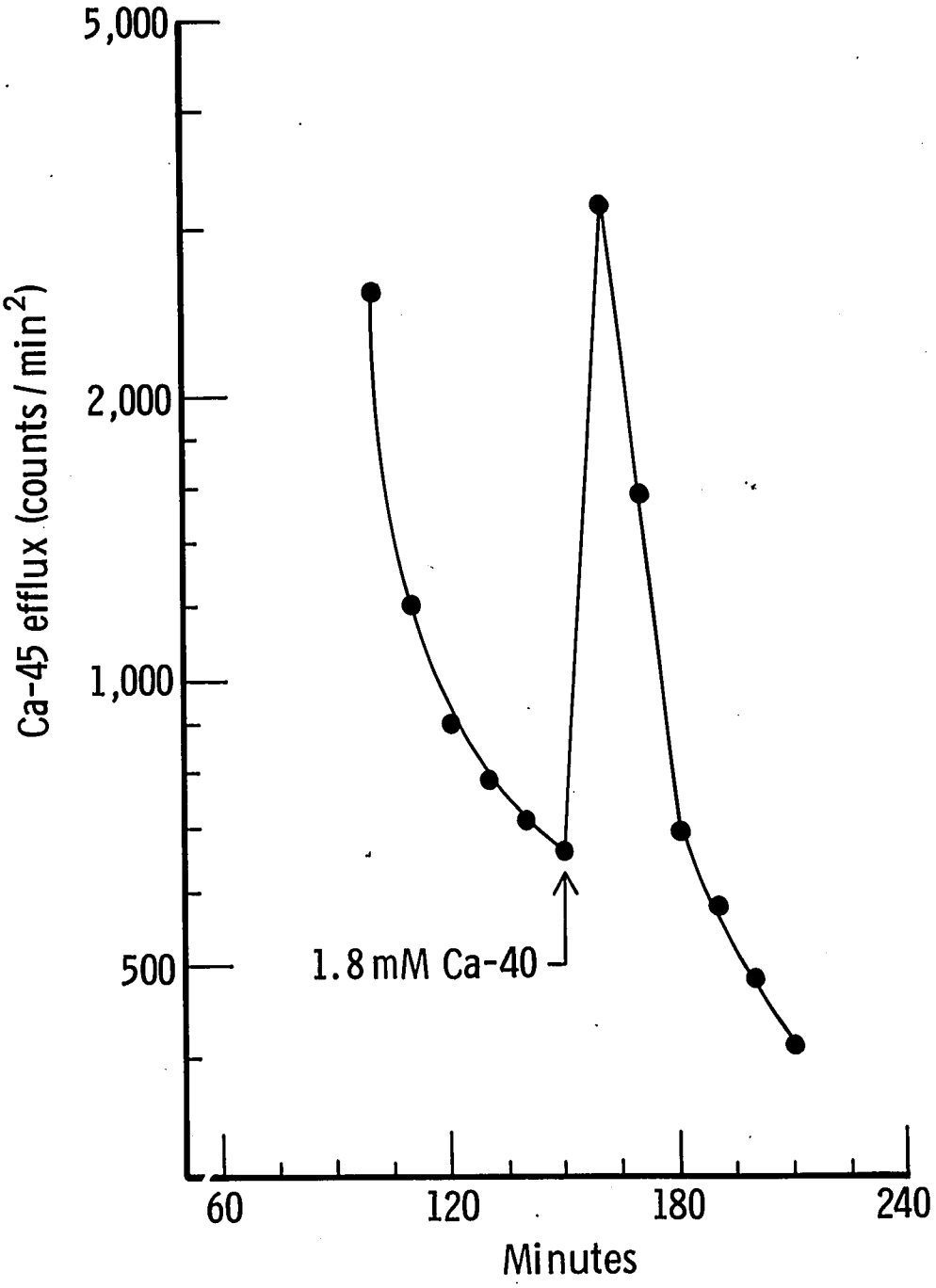


Figure 27. Release of Ca-45 from desheathed nerve upon addition of 1.8 mM Ca to the nerve bath. Nerves were soaking in Ca-free Ringer's up to the time of addition of Ca. Each point is the mean of 6 separate experiments.

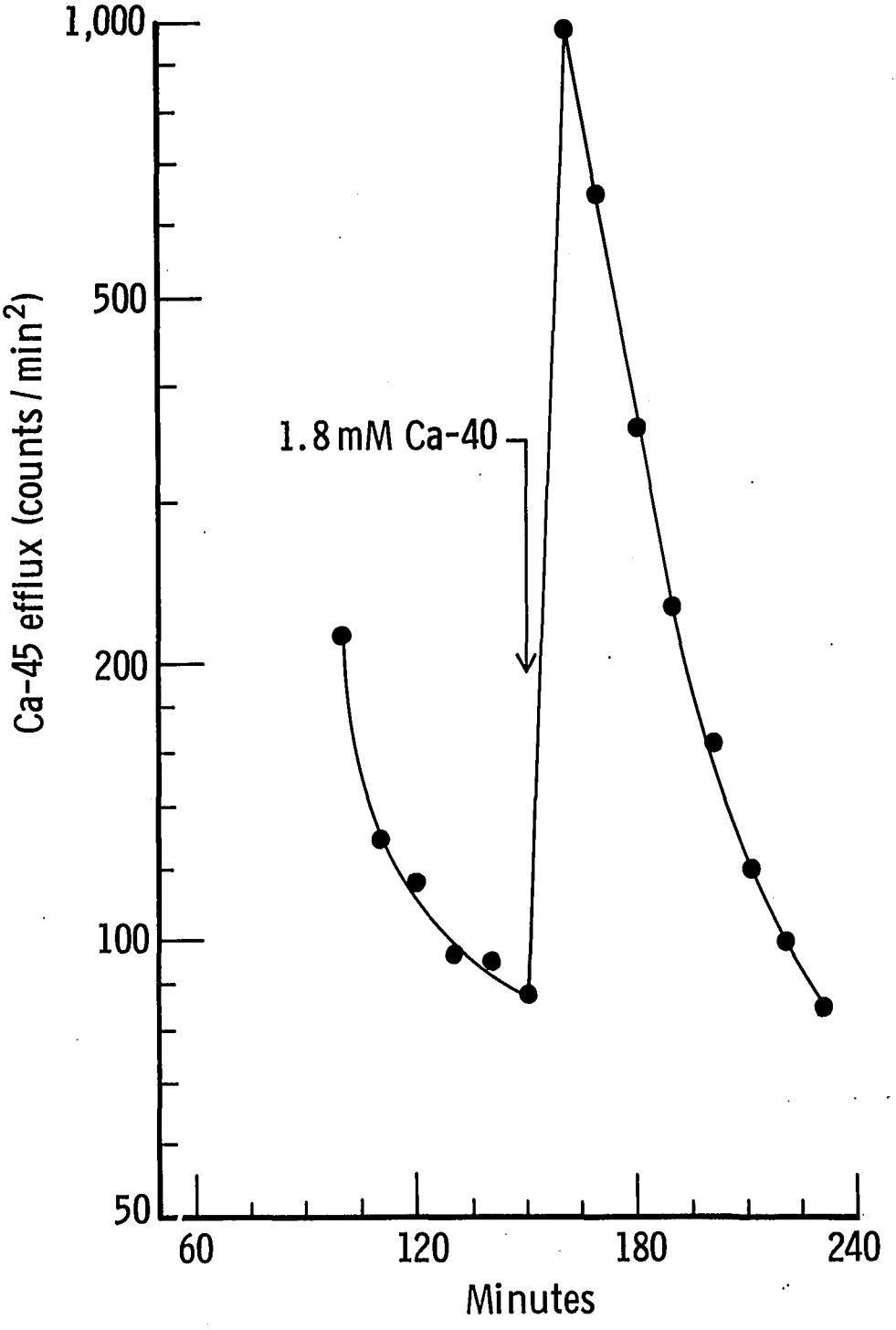


Figure 28. Release of Ca-45 from isolated sheaths upon addition of 1.8 mM Ca to the bath. Sheaths were soaking in Ca-free Ringer's up to the time of addition of Ca. Each experiment was carried out with 3-4 sheaths, and each point is the mean of 6 separate experiments.

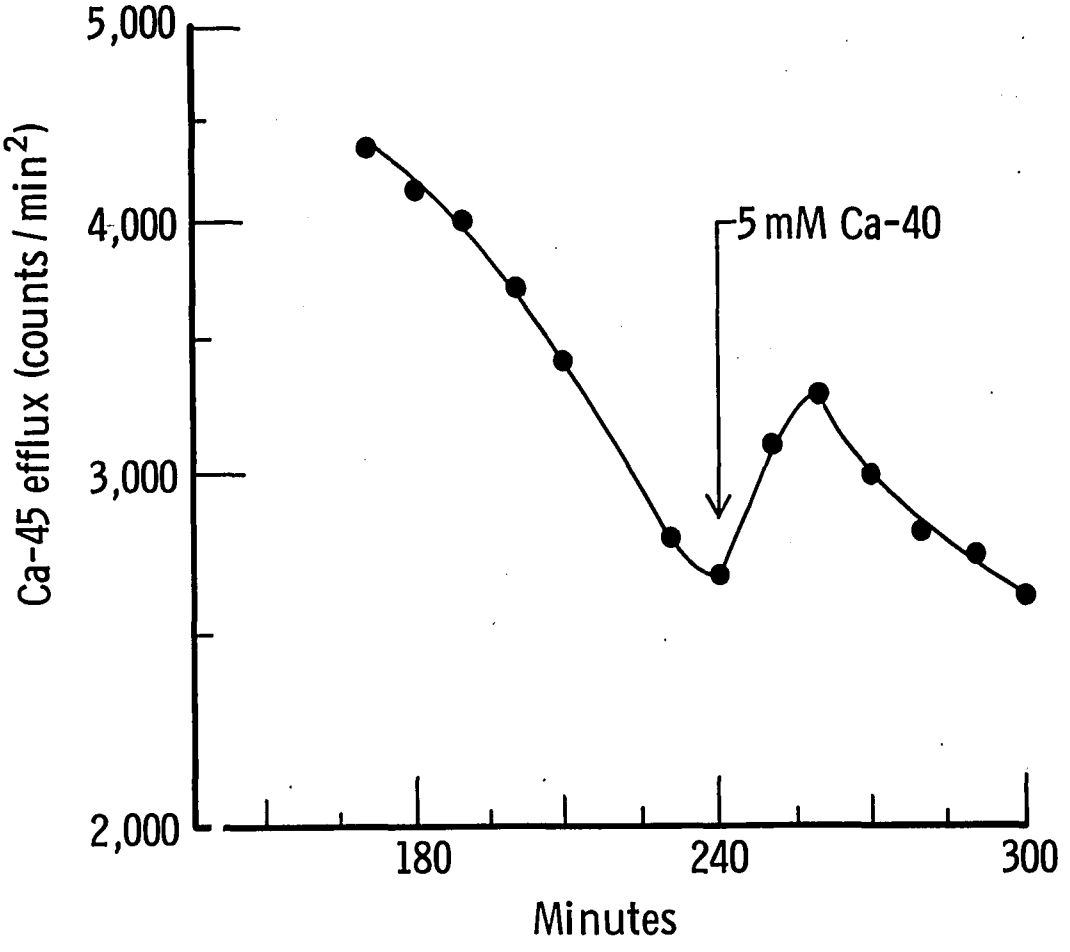
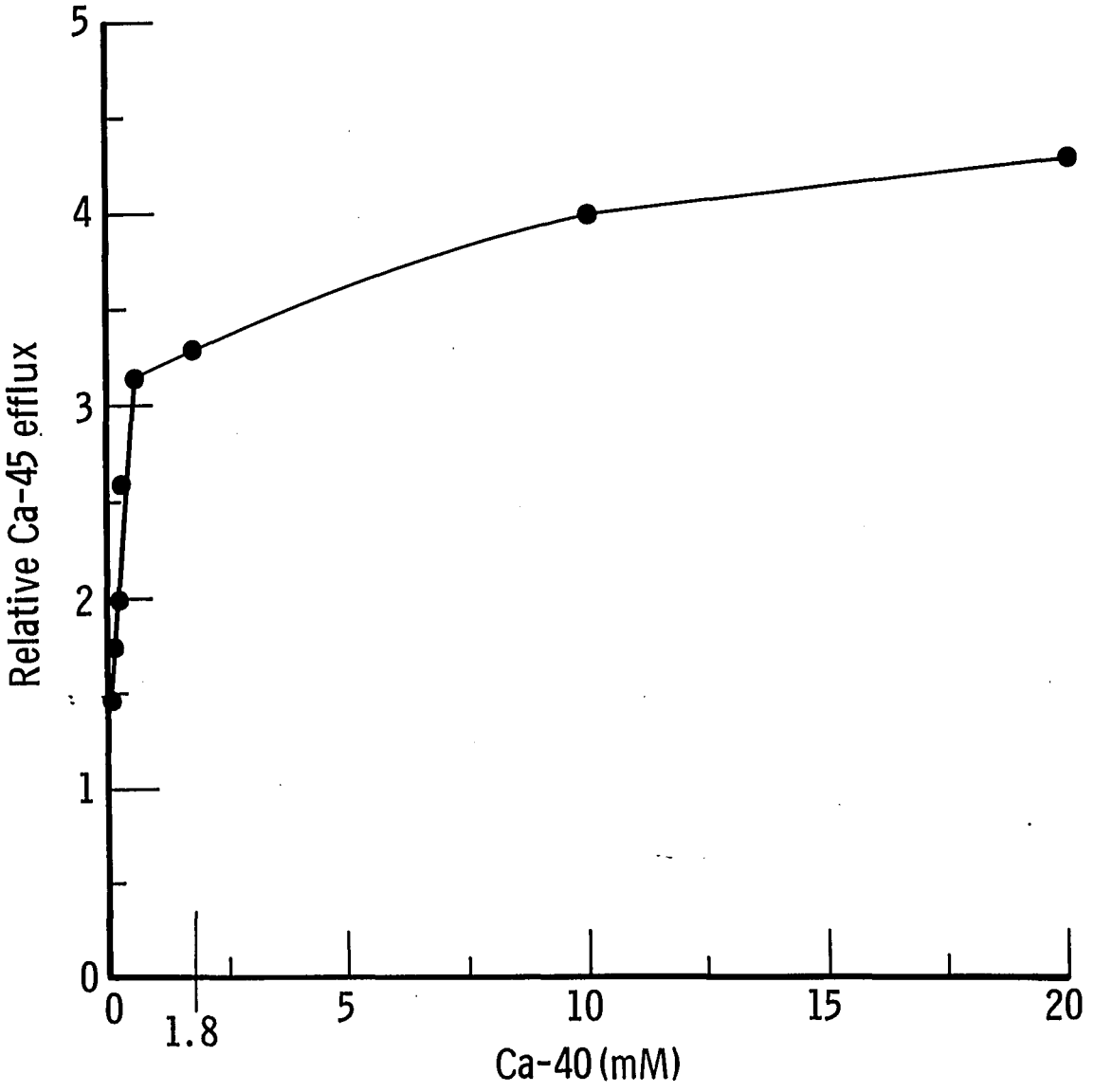


Figure 29. Release of Ca-45 from ensheathed nerve upon the addition of 5 mM Ca to a normal Ringer's solution (1.8 mM Ca). Each point is the mean of 6 separate experiments.

Figure 30. Relationship between the concentration of Ca added to the ensheathed nerve bath and the relative Ca-45 efflux at the peak release of Ca-45. The data for this graph was obtained from experiments like that of Fig. 26. In these experiments, concentrations of Ca ranging from 0.01 to 20 mM were added to an effluent solution that was Ca-free up to that time. The "saturation" effect appears at 0.45 mM Ca. Each point is the mean of 6 separate experiments.



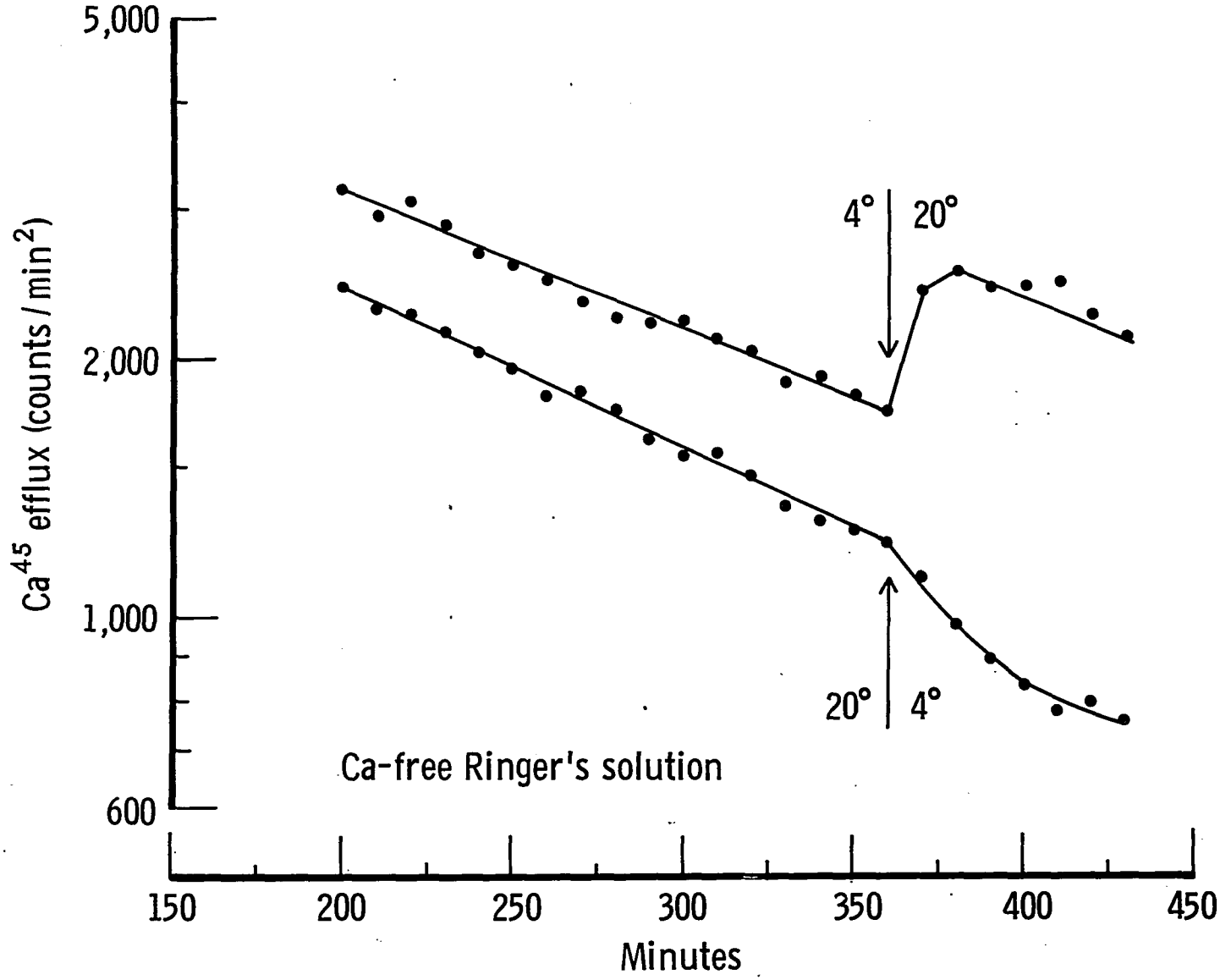


Figure 31. Influence of temperature on the time course of Ca-45 efflux from ensheathed nerve into Ca-free Ringer's. Each point is the mean of 6 separate experiments.

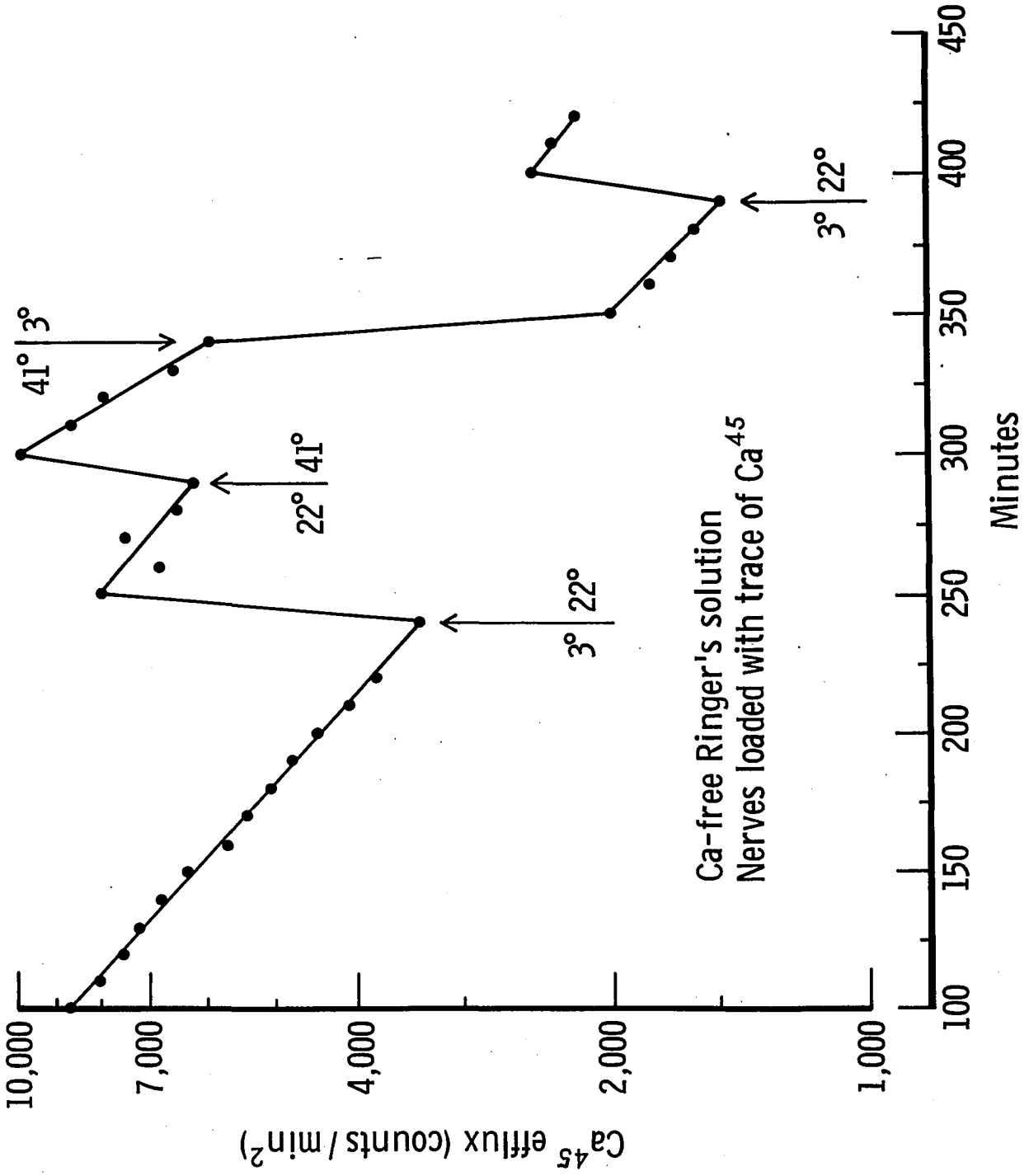


Figure 32. Influence of repeated temperature changes on the time course of Ca-45 efflux from ensheathed nerve into Ca-free Ringer. Each point is the mean of 6 separate experiments.

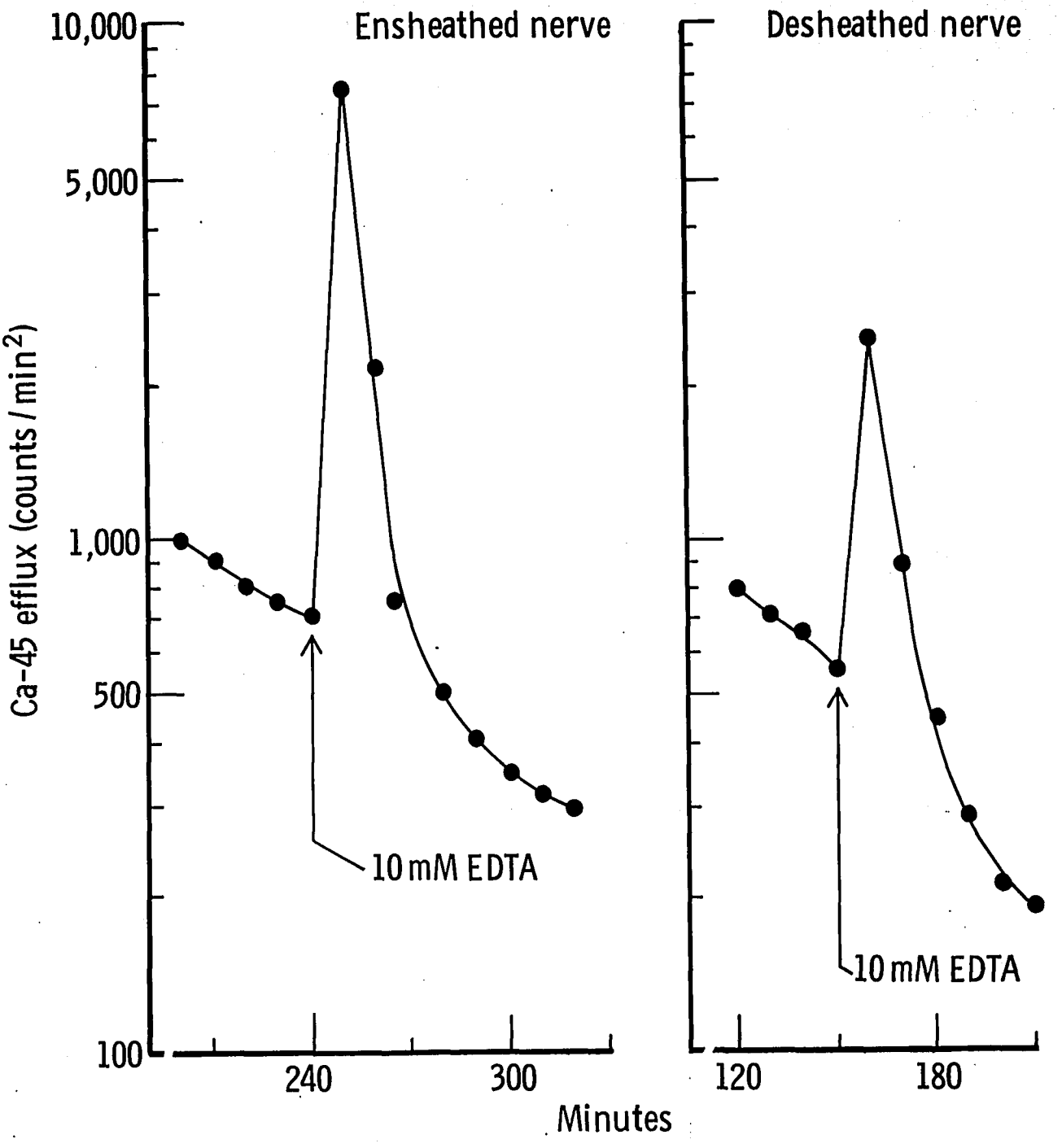


Figure 33a. Effect of EDTA on the time course of release of Ca-45 from ensheathed or desheathed nerve into Ca-free Ringer's. Each point is the mean of 6 separate experiments.

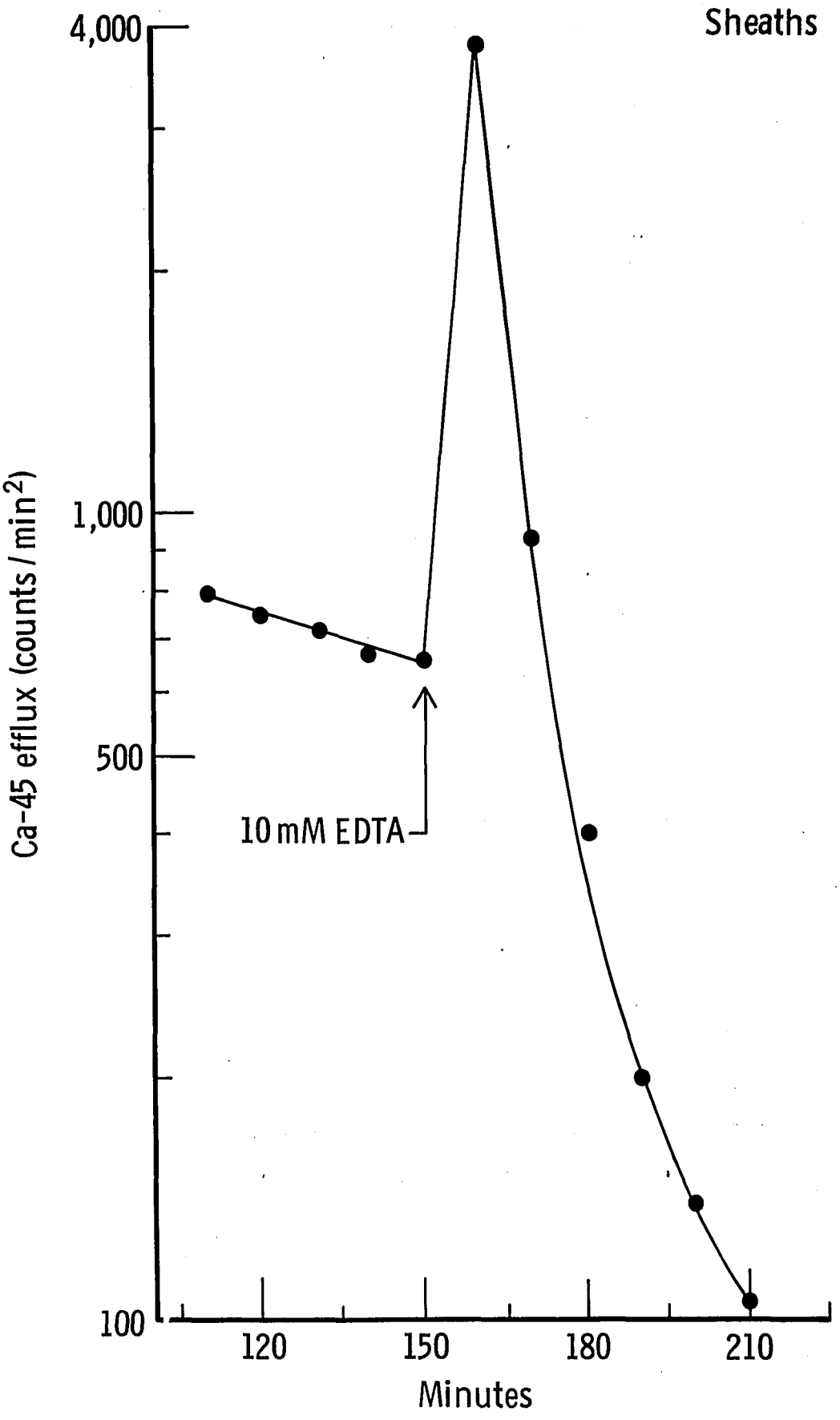


Figure 33b. Effect of EDTA on the time course of release of Ca-45 from isolated sheaths into Ca-free Ringer's. Each experiment was carried out with 3-4 sheaths, and each point is the mean of 6 separate experiments.

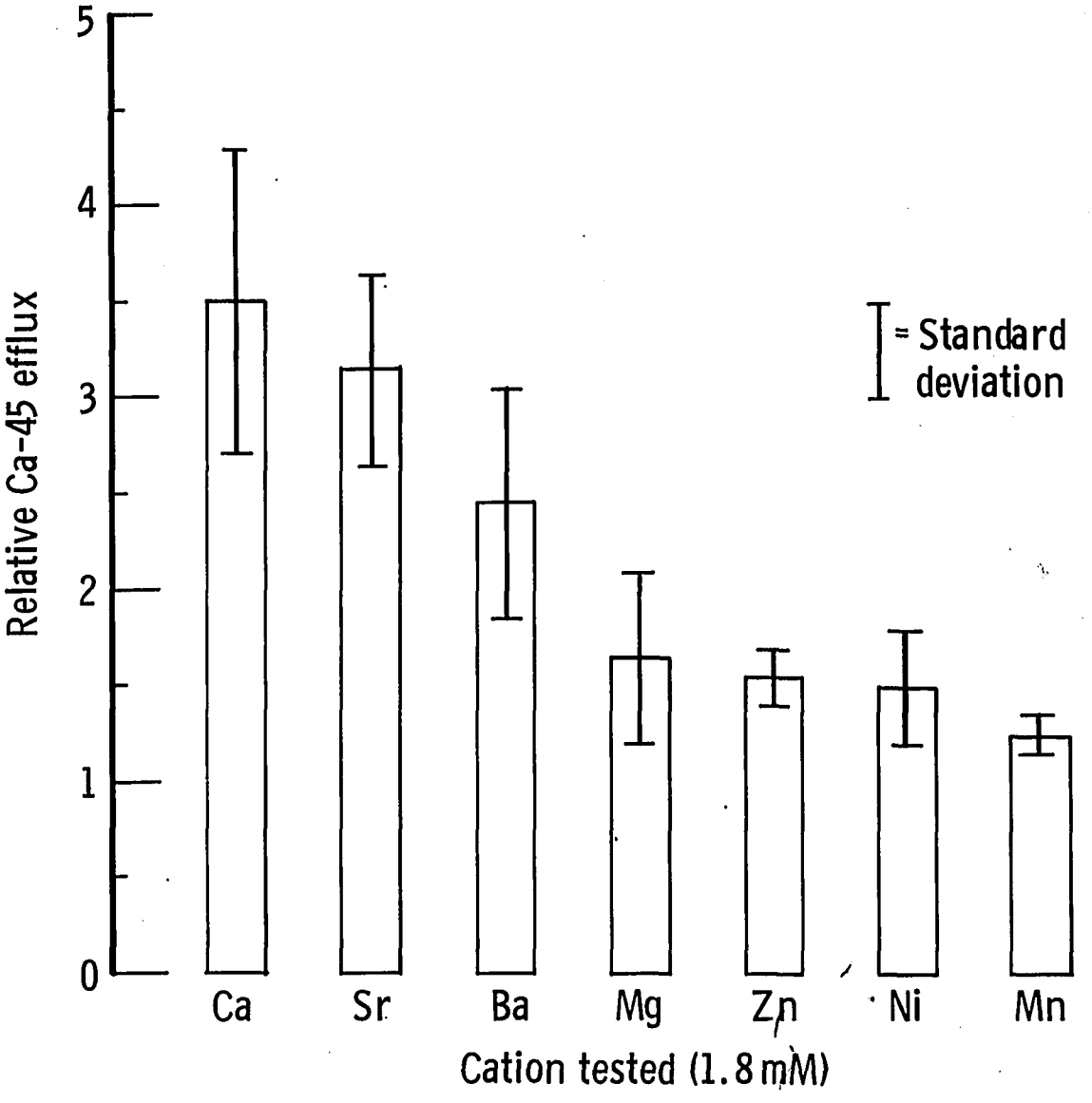


Figure 34. Release of Ca-45 from ensheathed nerve by 1.8 mM of Ca and other divalent cations. Each cation was added to the nerve bath after the nerve had been soaking in a Ca-free Ringer's for 240 minutes. Each bar represents the mean peak relative Ca-45 efflux (± S.D.) of 6 experiments. The order of potency is: Ca=Sr > Ba > Mg=An=Ni > Mn.

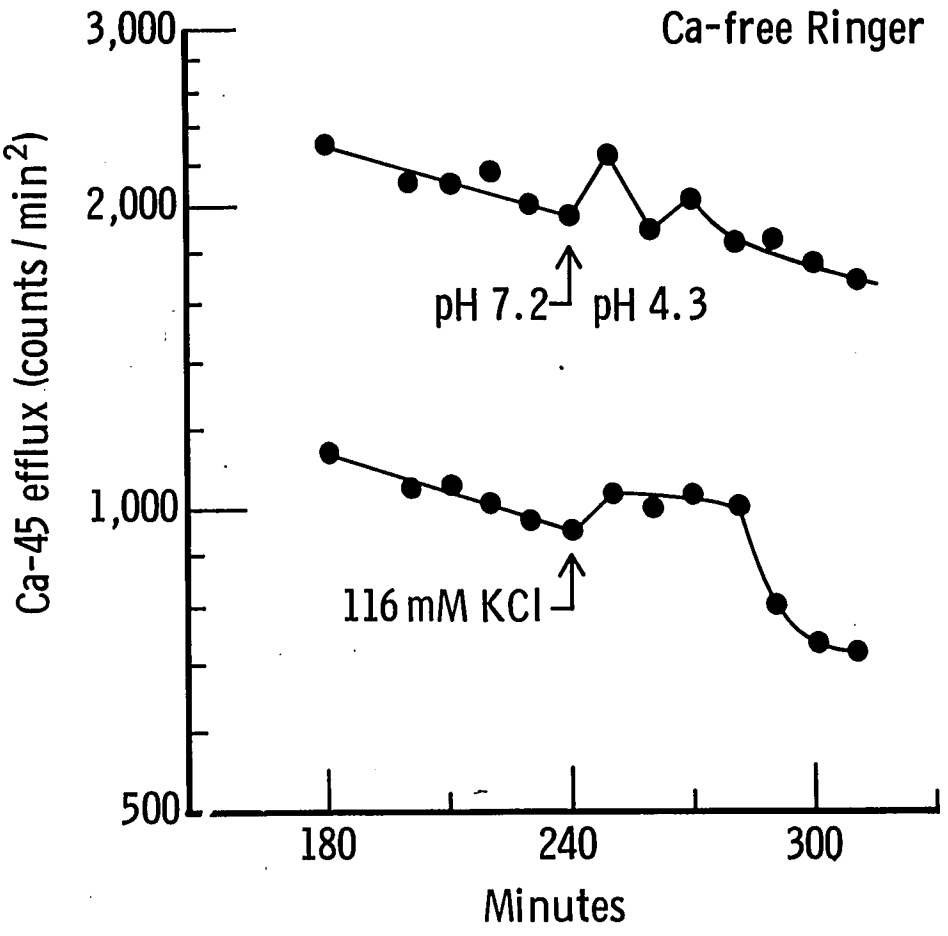


Figure 35. Effect of pH change and isotonic KCl on the time course of release of Ca-45 from ensheathed nerve into Ca-free Ringer's. The pH was changed from 7.2 to 4.3 by the addition of HCl to a Ca-free Ringer's. In separate tests it was shown that pH 4.3 has no effect on nerve excitability. Isotonic KCl produced a 10-15 mV depolarization of ensheathed nerve within a 30-minute period. Each point is the mean of 6 separate experiments.

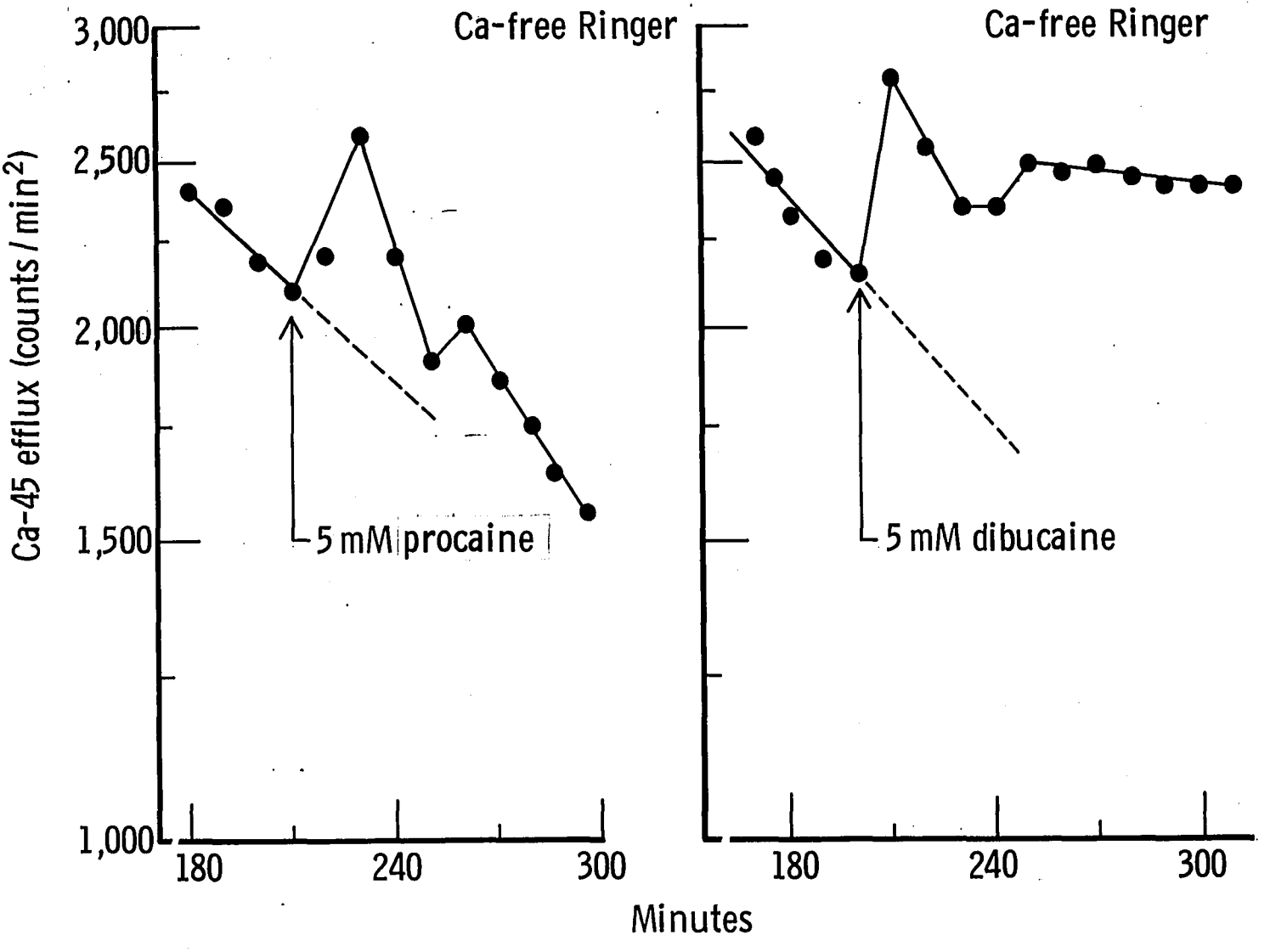


Figure 36a. Effects of procaine and dibucaine on the time course of efflux of Ca-45 from ensheathed nerve into Ca-free Ringer's. Treatment with the local anesthetic began at 200 minutes. Each point is the mean of 6 separate experiments.

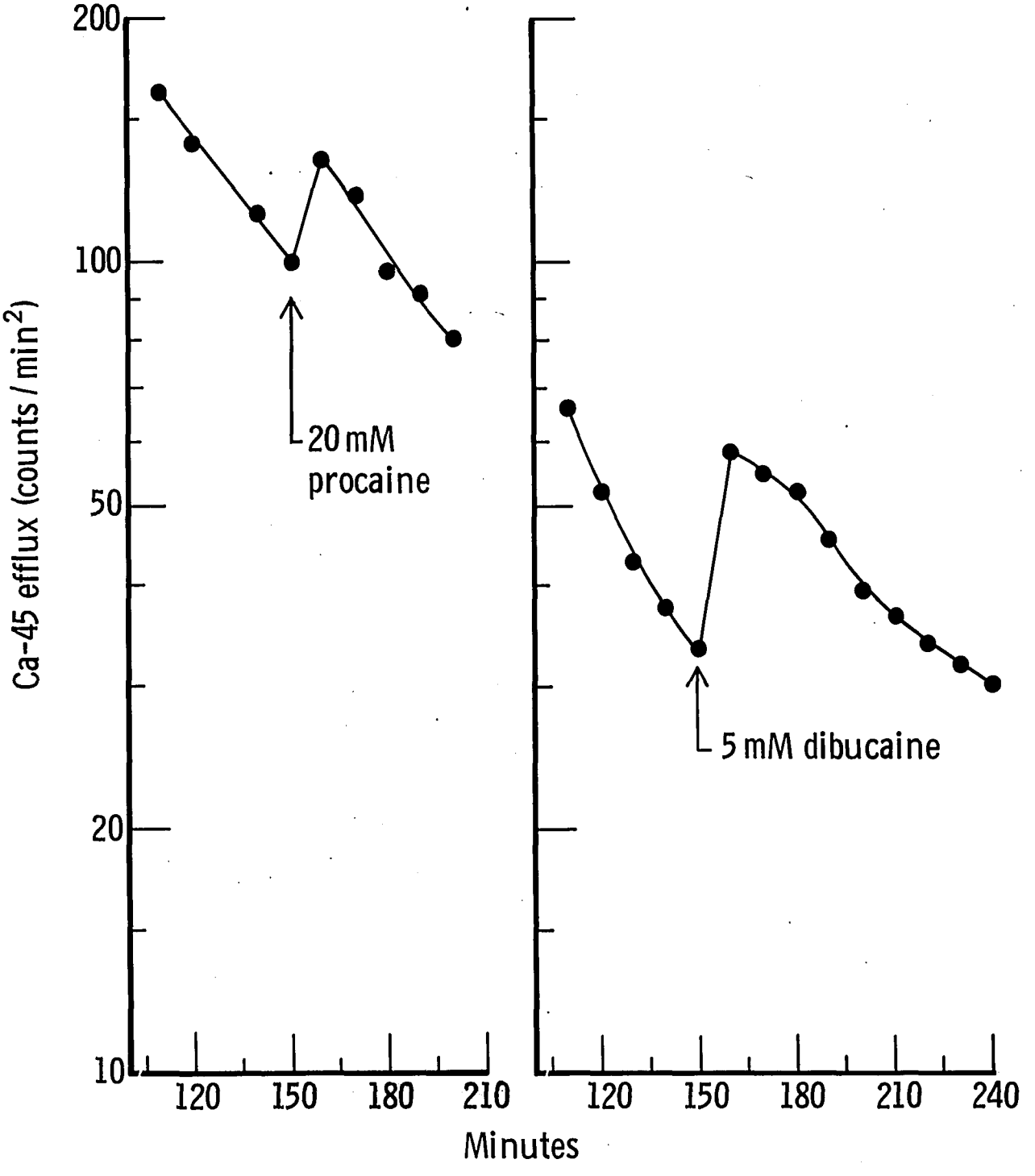


Figure 36b. Effects of procaine and dibucaine on the time course of efflux of Ca-45 from isolated sheaths into Ca-free Ringer's. Each washout experiment was carried out with 3-4 sheaths, and each point is the mean of 6 separate experiments.

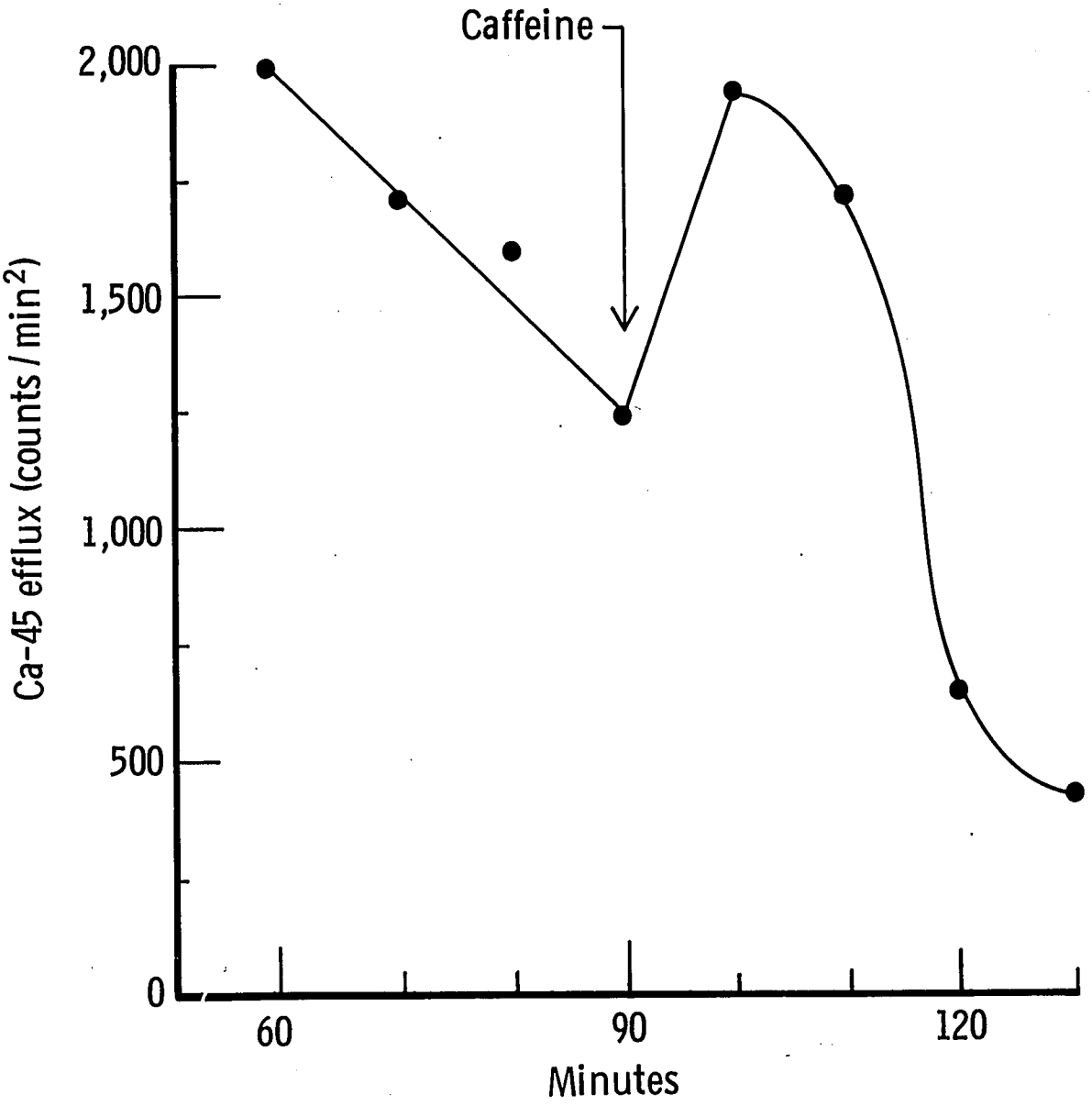


Figure 37. Effect of caffeine on the time course of efflux of Ca-45 from desheathed nerve into Ca-free Ringer's. Caffeine concentration was 20 mM, and it was added to the nerve bath at 90 minutes. Each point is the mean of 3 separate experiments.

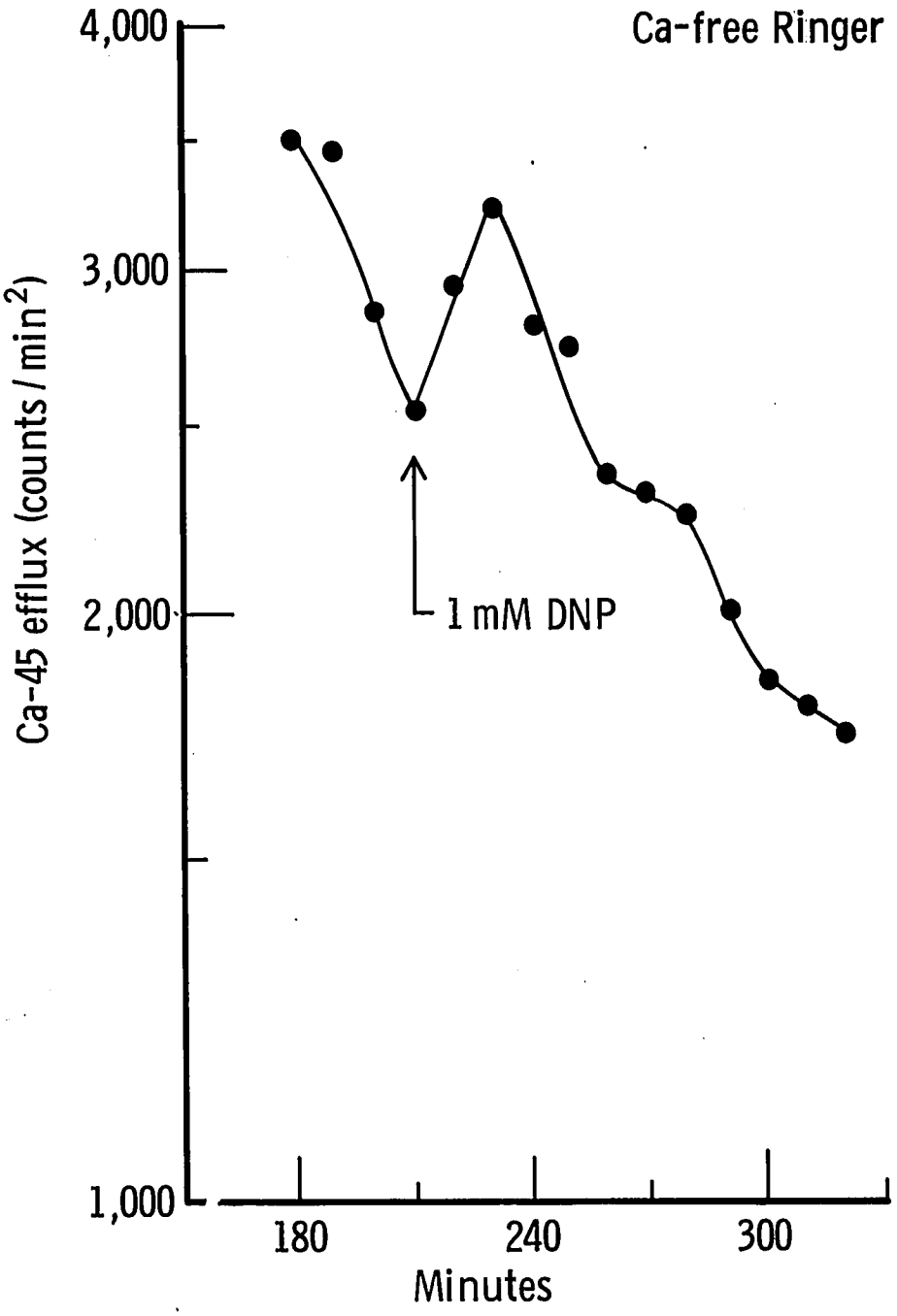


Figure 38. Effect of DNP on the time course of efflux of Ca-45 from ensheathed nerve into Ca-free Ringer's. Treatment with DNP began at 210 minutes. Each point is the mean of 6 separate experiments.

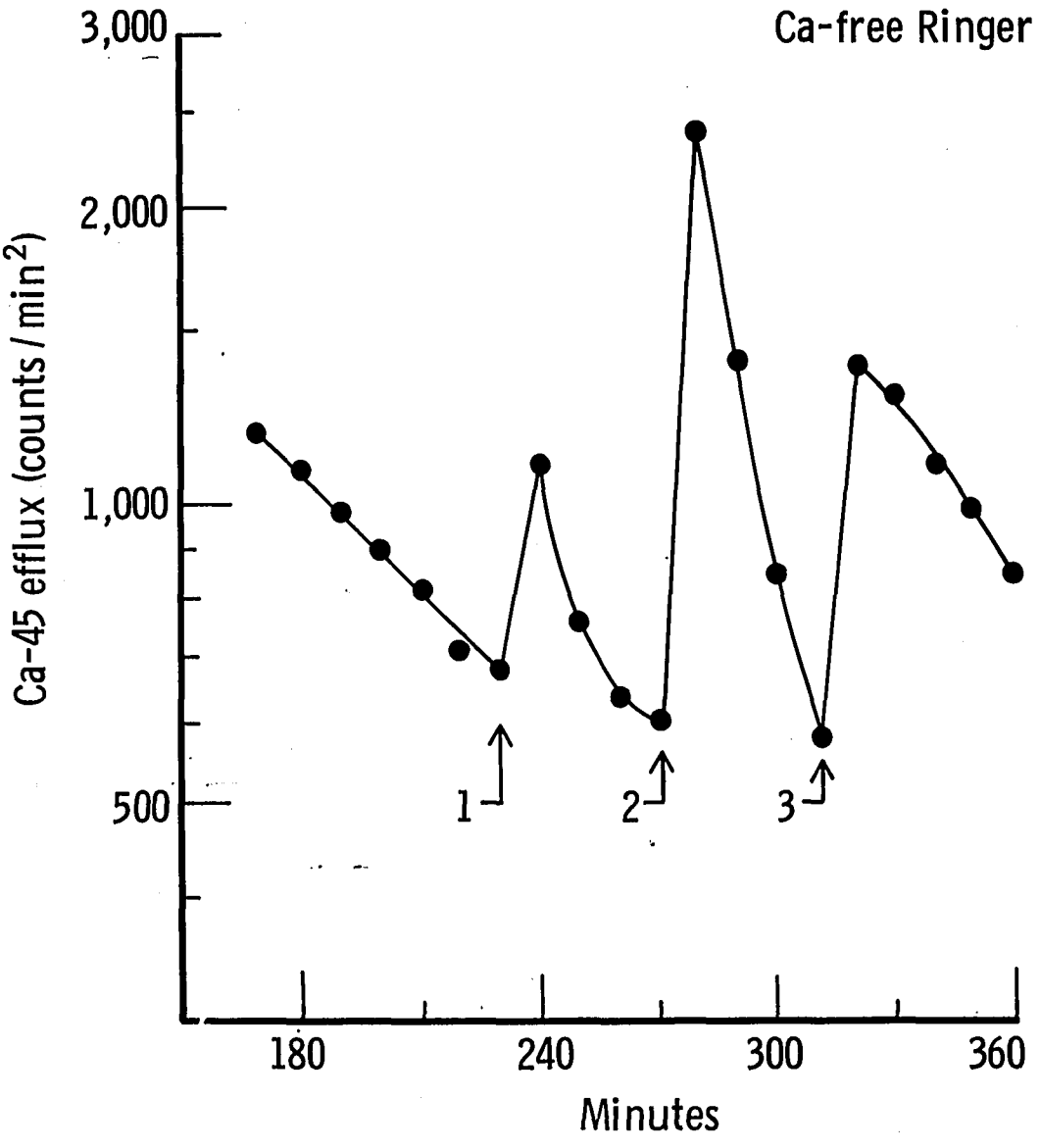


Figure 39. Effects of electrical stimulation on Ca-45 release from ensheathed nerve into Ca-free Ringer's. Nerve was placed across bipolar platinum electrodes directly in the collection fluid. In each case, square wave pulses of 80V and 0.1 milliseconds were applied. In trial 1 for 5 minutes at a frequency of 5 per second; in trial 2 for 5 minutesat a frequency of 20 per second; in trial 3 for 30 minutes at a frequency of 20 per second. Each point is the mean of 6 separate experiments.

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