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Method for assay of cholinesterase activity in skeletal muscle

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A METHOD FOR ASSAY OF CHOLINESTERASE ACTIVITY IN SKELETAL MUSCLE

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Introduction

In recent years work in this laboratory* upon dystrophic mice, Bar Harbor Strain 129, has led to the belief that at least a portion of the explanation for dystrophica myotonia may be explained by an analogy to denervated muscle.[1,2] This certainly is contrary to commonly accepted views of the disease. It was felt that assay of cholinesterase activity comparatively in dystrophic, denervated and normal muscles might help to explain the findings and the analogy. Upon reviewing the methods available for such a study, it was found that there were three glaring faults with most of the methods which made them unfit for such a study. The first was that many methods were not sensitive enough in the crucial step, the determination of acetylcholine after its reaction with the enzyme preparation. The second was that the method was not adaptable for use with the entire muscle, but instead determined localized concentrations of the enzyme. The third fault was that some of the methods were not adaptable for use with tissue homogenates which might interfere with the methods of determination, e.g. photometry.

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The purpose of this study was to develop a new method correcting these faults, yet retaining the properties of accuracy and reproductibility. The purpose of this paper is to present a review of other methods for acetylcholine and indirectly cholinesterase determination, present the new method in detail and to show by experimental trial and results that the method accomplishes these purposes.

Review of Other Methods

The methods for acetylcholine and indirectly cholinesterase determination are many. The ones listed below are by no means all, but only selected representative methods which have been used by various investigators with some success. Only a brief discussion of each is presented. For further details readers are referred to the original articles. The methods can best be discussed in outline form.

I. Chemical methods

A. B-carbonaphthoxycholine as substrate[3]

This is a colorimetric method whereby the substrate is hydrolyzed to B-naphthyl carbonic acid

which decarboxylates to B-naphthol spontaneously. This product is then coupled to a diazonium salt to form an azo dye extractable with ethyl acetate and can be measured by colorimetry. The biggest objection to this method is the use of a non-specific substrate and ramifications thereof.

B. Reaction of residual acetylcholine with hydroxylamine to form acethydroxamic acid[4]

This forms a soluble red-purple complex with ferric ions in acid solution and may be determined photometrically. This method works best for routine blood cholinesterase determination, but has been adapted for use with small amounts of tissue, e.g. 50 micrograms of nervous tissue. A major problem would be ridding the color solution of all components of muscle so accurate photometric determinations could be made.

II. Methods utilizing carbon dioxide production

A. Technique with the Warburg apparatus[5,6]

In this method cholinesterase containing tissue is incubated with known amounts of acetylcholine at constant temperature with constant shaking. This

hydrolysis of acetylcholine yields acetic acid and choline. This reacts with bicarbonate to form carbonic acid which decomposes into water and carbon dioxide. The amount of CO_2 is measured manometrically and can be used as an index of cholinesterase activity. This has probably been the most widely used method but is quite lacking in sensitivity.

B. Cartesian diver technique[7,8]

This is an ultra micro method, very good and very sensitive. It utilizes the same principles as the Warburg apparatus but makes a much more sensitive measurement of CO_2 production. It is best suited for determinations on a single cell. Cells used in the technique have been; megakaryocyte, single dorsal root ganglion cells and single muscle fibers. It is too sensitive for adaption to larger masses of tissues as whole muscles and requires special equipment not available in our laboratory.

III. Techniques utilizing a change in pH

A. Measured with a pH meter[9]

The enzyme preparation reacts with acetylcholine in

a standard buffer solution for a given time, usually 1-2 hours. The pH is measured before, during and after. The rate of change of pH is an indicator of enzyme activity. This is satisfactory only when the rate is directly proprtional to the enzyme activity. Important considerations in this method are that cholinesterase activity decreases with decreasing pH, the possible effect of the enzyme preparations on the buffer capacity of the system and the wide pH range of the system - usually 6 to 8. Later methods use smaller pH ranges (8.2 to 7.8) for a shorter time (200 seconds), but when the results are obtained many correction factors must be used and assumptions made which may not always be valid. The method is not highly sensitive.

B. Indicator measurement of pH change[10]

This utilizes the same principles as the pH meter, but involves the use of the photometer to measure color change in the indicator solutions. The two methods are comparable. They are both suitable for use on routine blood and plasma analysis, but

their accuracy for experimental work is questionable.

IV. Utilization of titration to maintain a fixed pH[11,12]

This method consists of titrating the enzyme-substrate reaction mixture with NaOH to maintain a fixed pH, and the NaOH used is a measure of the enzyme activity. The most critical portion is of course determining the pH and keeping it constant enough so that results are reproducible. The pH is measured either by an indicator or preferably by electrometric methods. It is not an especially sensitive method.

V. Spectrophotometric method[13]

This consists of measuring the hydrolysis of the substrate at 24 millimicrons. There is not a good correlation of results with gasometric methods if acetylcholine is the substrate, but results correlate well if benzoylcholine is used as the substrate. It has proven to be a bit more sensitive than gasometric methods.

VI. Determinations using non-specific substrates[14]

These are many and varying including procaine, sal-

icylic acid esters, phenyl esters, tributyrin and nitrophenyl esters. Their use requires purified enzyme preparations with specificity known and are thus not satisfactory for use with tissues.

VII. Histochemical techniques[15]

This technique was originated by Koelle and consists of incubation of teased preparations or frozen sections of muscle with acetylcholine and copper glycinate. It results in the formation of copper thiocholine at the site of hydrolysis of acetylcholine. The mixture is saturated with copper thiocholine to prevent diffusion from the sites of reaction. Then the material is reacted with ammonium sulfide which converts the white precipitate to the dark brown precipitate of sulfide. The tissue is then fixed, mounted, counterstained and read microscopically for degrees of precipitation. Faults of the method are that it involves only a portion of the whole muscle, that it may not be sensitive enough to show smaller amounts of cholinesterase than found at sites such as the myoneural junction and that it requires sub-

jective estimation for quantity of reaction.

VIII. Biological assay methods - these are simply listed

A. Frog heart[16]

B. Guinea pig intestine[17]

C. Mouse intestine[18]

D. Leech muscle[19]

E. Frog rectus abdominus muscle[20]

F. Cat's blood pressure[21]

G. Venus mercenaria heart[22,23]

All of these assays utilize incubation of known amounts of acetylcholine with the enzyme containing tissue and estimating the degree of loss of effect between nonhydrolyzed controls and the enzyme-substrate preparations. The most sensitive and most specific of the assay methods is the use of Venus mercenaria hearts. This preparation is least interfered with by extraneous materials in tissue such as histamine, serotonin, potassium and calcium.[23] For these reasons, and because of prior experience with this method in our laboratory, this is the method chosen for this study.

Description of the Method

A. Enzyme-substrate preparation and reaction

The animals chosen for the study were white mice, littermates of approximately equal size and weight. One-half of the animals were chosen at random, anesthetized and a 2 mm. section of the sciatic nerve in one leg removed. The other half of the litter was used for controls. The gastrocnemius-soleus muscle group was removed from each leg, immediately frozen with dry ice and stored for a short period at -15°C until ready to process. The next step was to smash the muscles while frozen in a stainless steel cylinder (see figure 1), which shattered them to facilitate homogenization. The muscles were then weighed quickly after transfer to the homogenizing flask and equal amounts of calcium free Ringer's bicarbonate added to each specimen. Tissue homogenates were then made of the muscles by a Vir-Tis rotary blade homogenizer. The homogenate was filtered to remove connective tissue not cut by the blades and the weight of this tissue subtracted from the original muscle weight.

The remainder was considered to be essentially the weight of actual muscular tissue. Then an equal aliquot of each muscle was transferred to a Warburg incubating flask. The aliquot contained muscle with its weight known to the nearest 0.5 milligram. To each homogenate equal volumes of calcium free Ringer's bicarbonate were added to bring the whole volume to 4 cc. A standard amount of acetylcholine bromide (0.3 cc. of 1 x 10^{-4}M) was placed in the side arm of the flask. The apparatus was sealed and contents equilibrated at 37.5°C with 95% N_2 for ten minutes. The substrate and enzyme were then combined and incubated with constant shaking by the Warburg apparatus. The gas produced by the reaction was used to determine the rate of reaction which would rise rapidly, plateau and then fall more slowly. End point of the reaction for this experiment was chosen at the end of the plateau, about one hour of incubation. The reaction was stopped by diluting the samples with equal amounts of artificial sea water containing 1 mgm/cc. of neostigmine which inhibited the cholinesterase. Controls of acetylcholine alone in

equal dilution were incubated with each pair of muscles to use as reference standards for assay on the Venus heart. The samples and controls were stored at 4°C until ready to assay.

After allowing 14 to 28 days for changes of denervation, the gastrocnemius muscles of the remaining mice were prepared in the same manner using the normal leg of the mouse for the control. Proof of denervation was the demonstration of spontaneous fibrillation in the involved muscles. No attempt was made to allow for the lesser weight of the denervated muscle in preparing the homogenate for incubation because it was felt that any error in using a greater proportion of the denervated muscle would tend to err in favor of a greater cholinesterase activity in these muscles than in normals, and conversely if less activity were shown in the denervated specimens that it would make the results even more significant.

B. Assay of the Preparation

Clams of the species Venus mercenaria were obtained from the Woods Hole laboratories in Maine. They were

shipped by air, packed in ice, in lots of 24. Upon arrival they were placed whole in a bath of artificial sea water made according to the recipe of Tower and McEachron[23] and stored at 4°C. To prepare the heart for assay the shell of the clam was broken with a hammer and the mantle stripped away exposing the pericardial sac. This was opened and silk ligatures were placed around each end of the single ventricle leaving as large a contractile portion of the ventricle as possible. One ligature was used to anchor the heart to a hollow glass stem through which O_2 was bubbled to oxygenate the bath. The remaining ligature was fastened to a long but light and carefully balanced lever arranged to record contractions on a smoked kymograph paper (see figure 2). The heart was suspended in a 30 cc. bath of artificial sea water maintained at a constant temperature of 14°C, the temperature at which the heart proved to be most efficient and lasted for the longest time. With the constant bubbling of O_2 and frequent bath changes, a single heart often lasted for periods of 8 to 24 hours.

The bath was a specially designed glass bowl into which diluting sea water could be run with the turn of a stopcock and when a volume of 100 cc. was reached the bath was automatically siphoned back to 30 cc. Then after a given substance was added to the bath and its effect upon the heart recorded, it could, by washing the bath three times, be diluted to infinitely small concentrations and no longer have an effect upon the heart.

All of the hearts varied in sensitivity to acetylcholine, and their sensitivity was determined by assay of standard solutions of acetylcholine bromide. Most hearts would respond by a depression in amplitude of 1/3 when 0.1 ml. of 1×10^{-7} or 1×10^{-6} grams percent was added to the bath. The final concentration presented to the heart was then in the range usually of 3×10^{-10} grams percent. Some hearts were even sensitive to concentrations of 1.5×10^{-14}. A seasonal variation was noted in that the hearts were generally least sensitive in the early spring and most sensitive in late fall.

After sensitivity of each heart was determined, the samples to be measured were diluted to appropriate approximate concentrations. The control sample was assayed using various aliquots of the sample until a depression in amplitude of 1/3 to 1/2 was obtained. After careful washing and restabilization of the heart, equal amounts of the enzyme-substrate mixtures were assayed for remaining acetylcholine. After each sample had been assayed the heart was again checked with the acetylcholine standards to reveal any change in sensitivity. If a change was noted the samples were assayed on other hearts until one was found with sensitivity unchanged.

Controls were run to see the effects of prostigmine K^+, Ca^+, muscle alone, serotonin and histamine. Prostigmine had no effect nor did the muscle homogenate alone. Serotonin and histamine both had a stimulatory effect upon the heart manifested by an increase in stroke amplitude. K^+ and Ca^+ had no effects in doses much higher than could possibly be present in the samples.

The hearts were noted to respond to acetycholine both by a decrease in amplitude and by a decrease in

rate but the former was predictable and reproducible and was, therefore, used for measurement. The hearts responded to acetylcholine in a logarithmic fashion, that is twice the concentration produces about 1½ times the depression. Because many determinations with controls on each heart would be necessary to establish the dose response curve and a long series done on a series of hearts to get a basically sound curve to use in general, no attempt was made at this time to estimate concentrations of acetylcholine in unknowns by amount of depression. I do feel, however, that with more experience and the long series of determinations mentioned, that it would be quite possible to quantitate this determination and even closely estimate the total cholinesterase activity in skeletal muscle. Figure 3 below illustrates a typical record of an assay on the heart.

Results:

For reasons of simplicity it was decided to report the results of the assay in percentage depression of the heart amplitude by the various samples. For reasons already stated quantitation of the acetylcholine content of the samples was not done.

The first portion of the experiment was to see if equal samples of each gastrocnemius-soleus muscle group of the mouse prepared by the above method would prove to have equal effects upon the clam heart within the range of reasonable laboratory error. Results are shown in Table 1.

Trial No.	Control %	Left Leg %	Right Leg %
1	34	12.1 - 60 mgm.	12.1 - 60 mgm.
2	37	10.4 - 60 mgm.	10.2 - 60 mgm.

Table 1. Left leg and right leg are of the same mouse assayed upon the same heart. Assay reported as % depression in amplitude of the heart by equal aliquots of the samples. The second figures in each box represent the weight of the muscle homogenate used.

In each of the instances above 60 mgm. of normal mouse muscle was incubated with acetylcholine. Trials 1

and 2 are assayed on different hearts with different sensitivities to explain the differences in % depression.

The next portion of the experiment was to compare the activities of denervated muscles to activities of the opposite leg normal muscles. Results are shown in Table 2.

Trial	Control %	Normal Muscle %	Denervated Muscle %
1	30.6	20.0 - 14.6 mgm.	27.8 - 14.6 mgm.
2	27	17.1 - 14.6 mgm.	25.7 - 14.6 mgm.
3	29.3	13.2 - 20 mgm.	26.1 - 20 mgm.
4	39.3	20 - 25 mgm.	30 - 25 mgm.
5	34.3	21.3 - 61 mgm.	33.8 - 61 mgm.
6	14.8	5.3 - 52.7 mgm.	13.6 - 52.7 mgm.
7	14.8	7.1 - 52.7 mgm.	13.0 - 52.7 mgm.

Table 2. This shows the results of comparative assay of equal aliquots of samples of normal and denervated muscles on the Venus heart. Shown are figures representing the % depression in amplitude of the heart and the weight of the muscle homogenate used in the incubation according to the method described above.

Table 3, below shows the results obtained when attempting to show that the heart does not respond

according to an arithmetic dose-response curve. These are the same samples as No. 6 and 7 assayed on another heart.

Trial	Con-trol	Dener-vated Muscle %	Normal Muscle %	2 Times Dener-vated Muscle %	2 Times Normal Muscle %	4 Times Normal Muscle %
1	20	16.1	8.2	22	13.0	18.2
2	20	18.2	9.2	22.8	13.6	18.4

Table 3. This represents the effect of doubling and quadrupling the amount of the samples being assayed. Figures again represent % depression in amplitude. It illustrates that the hearts do respond logarithmically to increasing doses of acetylcholine and also that the difference between cholinesterase activities in denervated and in normal muscles is greater than would be apparent from the figures in Table 2.

Thus, one can see that the % depression in response to sample of normal muscle in each trial is about ½ that of the denervated. When twice the amount of each sample is assayed the response is not anywhere near doubled. Also, it may be noted that the response to a single dose of the denervated muscle sample is barely duplicated by four times the dose of the normal muscle samples.

Discussion

The chemical mediator mechanism of skeletal muscle excitation is well established. Experimental evidence was culminated in 1934 by Dale and his coworkers,[24,25] who identified acetycholine as the chemical transmitter substance in skeletal muscle. In 1938 Marnay and Nachmansohn[26,27] demonstrated that acetylcholine is rapidly destroyed at the site of its action by an enzyme, cholinesterase. Without entering into a detailed discussion of the anatomy and physiology of the nerve-muscle junction and muscle itself, let it suffice to say that, in response to stimulation of a motor nerve, acetylcholine is released at the nerve ending and in turn reacts with receptor sites of the muscle sole plate. This reaction causes changes in the physiologic properties of the muscle fiber so that it contracts. In the area of this reaction there are normally high concentrations of cholinesterase which hydrolyze the acetycholine into acetic acid and choline almost immediately after its release. The response to the stimulatory effect of acetylcholine is thus quite short as is the contraction

phase of a muscle fiber. When a muscle fiber has been denervated many changes take place. The one that concerns this study primarily is that the fiber becomes much more sensitive to acetylcholine.[28-32] It is estimated to be about 1,000 times as sensitive.[33] Along with this well known fact has come the finding that curare-like substances have a decreased effectiveness upon denervated muscles.[34] Also, it has been noted that the effects of anticholinesterase drugs are markedly less upon denervated than upon normal muscles.[35] Denervated muscle has also been found to have an abnormal amount of spontaneous activity even while in the supposed resting state. This is best demonstrated by the use of concentric needle electrodes to record the electrical activity of these hyperactive cells. What is found is called spontaneous fibrillation and consists of spontaneously repeating depolarizations of single muscle fibers of low electrical amplitude at 8-10 times per second. This activity is augmented by the use of anticholinesterase drugs.[36]

The most logical explanation for these changes in

denervated muscle is that there is an abnormal increase of acetylcholine in the muscle, stimulating these muscle fibers even in the resting state. This is in the absence, of course, of neural stimulation for its release.[37] The increase in acetylcholine may be demonstrated by perfusion experiments.[37] Intra-arterial injection of acetycholine into fibrillating denervated muscle increases the fibrillatory activity.[38] In view of these findings the postulated reason for acetylcholine excess is a decrease in the activity of cholinesterase. It has been shown that there is a decrease in the cholinesterase activity in denervated muscle, specifically at the myoneural junction, and at other sites about the fiber as studied by several authors.[39] The work of Giacobini and Holmstedt[40] has shown that there are several sites in the muscle where cholinesterase is localized. They describe the enzyme at:

1. The motor end plate - here are found the highest concentrations in normal muscle.
2. The cup shaped area over the ends of muscle fibers - here is the second highest concentration of cholinesterase.

3. Muscle spindles - third highest in concentration.

4. Muscle fibers themselves - to quote the author is, "...the lowest cholinesterase content determined in any structure hitherto investigated by means of the diver technique."

It should be pointed out then that the studies mentioned above which reported decreased cholinesterase were histochemical methods which are not especially sensitive, so when they report decreases they are measuring only the first three areas. When the whole mass of the muscle is considered, in relation to these small structures where the enzyme is most concentrated, it becomes quite apparent that this enzyme contained in muscle fibers themselves may become quite important. When considering also the tremendous histologic change that takes place in a denervated muscle, the foremost of which is complete disappearance of the nerve fibers and thus the motor end plate, it is logical that the cholinesterase in the muscle fibers assumes a greater relative importance than in normal muscle. Therefore, it seems important, especially in denervated muscle to use the whole

muscle for cholinesterase determinations.

By the new technique described above it is felt that this purpose is accomplished. The results as shown in the various tables are unanimous in showing that cholinesterase activity in whole denervated muscles is markedly reduced when compared to whole normal muscles. The method is sensitive in terms of detecting very minute amounts of acetylcholine and can also detect very minute changes in acetylcholine concentration. The results are reproducible as demonstrated by the comparison of equal amounts of normal muscle.

The work for which the method was designed, to compare the cholinesterase activity in dystrophic muscle to that in denervated and in normal muscle is not yet completed, but it is felt that the method will be able to give an accurate picture of the comparative activities.

Summary

This paper presents a review of the majority of the presently used methods for determination of cholinesterase activity and acetylcholine determination. It then describes a biological assay method using the Venus mercenaria heart for determination of cholinesterase activity indirectly by its effect in hydrolyzing acetylcholine. It discusses the reasons why it is believed that this method is better than others for determination of cholinesterase activity in muscle in various disease states where it might be altered. The experimental trial for the method is to verify results of other workers in demonstrating a decrease in cholinesterase activity of denervated muscle. A significant decrease is shown.

Conclusions

1. The Venus mercenaria heart is a sensitive method for assay of acetylcholine.
2. The results of this method proposed for assay of cholinesterase activity are reproducible.
3. By this method, verification of findings by previous workers of a decrease in cholinesterase activity in denervated muscle has been obtained.
4. This method is a reasonable one to use for assay of cholinesterase activities of muscles in various disease states where they might be altered.

Figure 1. Diagram of the stainless steel cylinder used for smashing muscles as described.

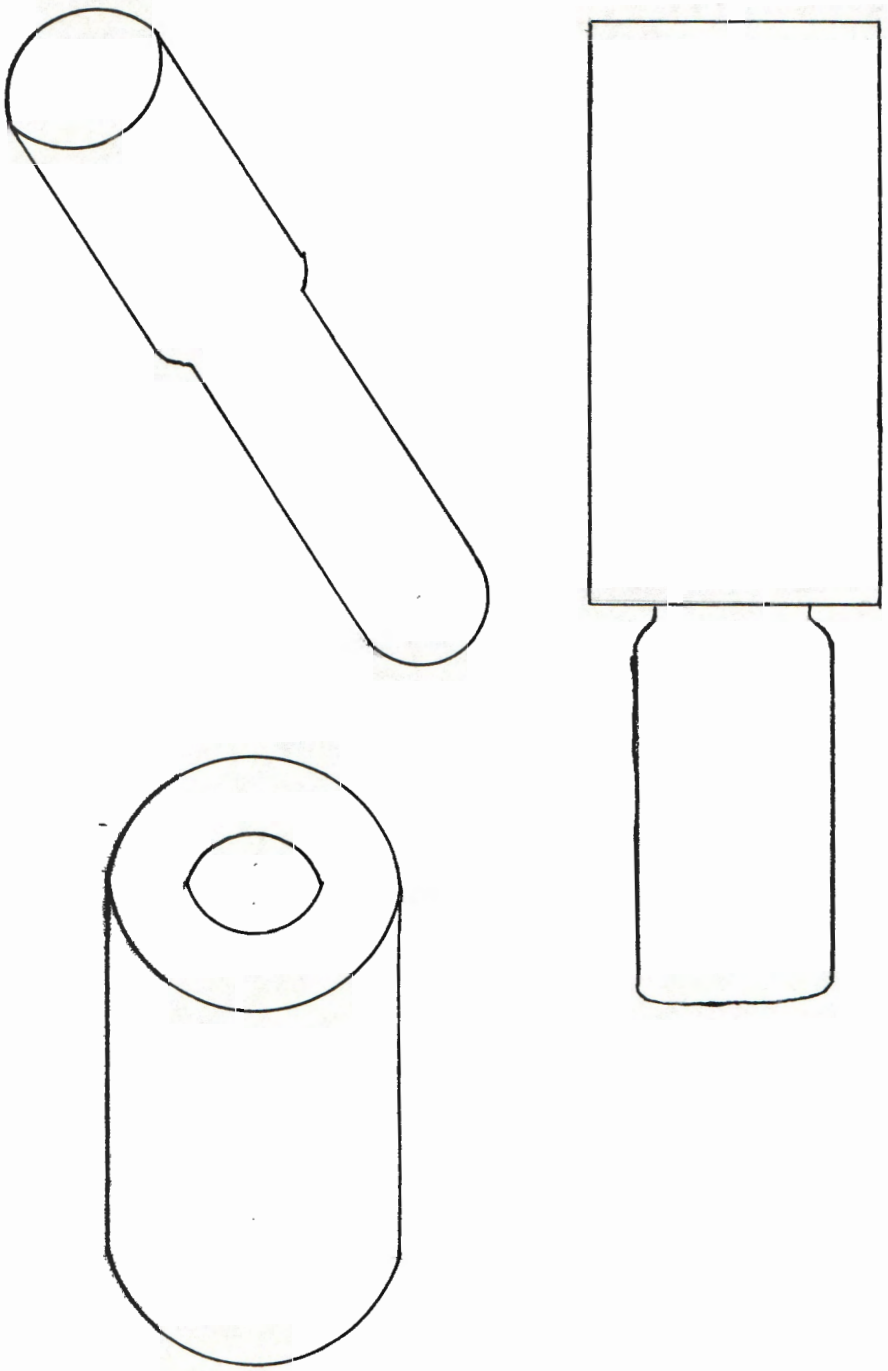
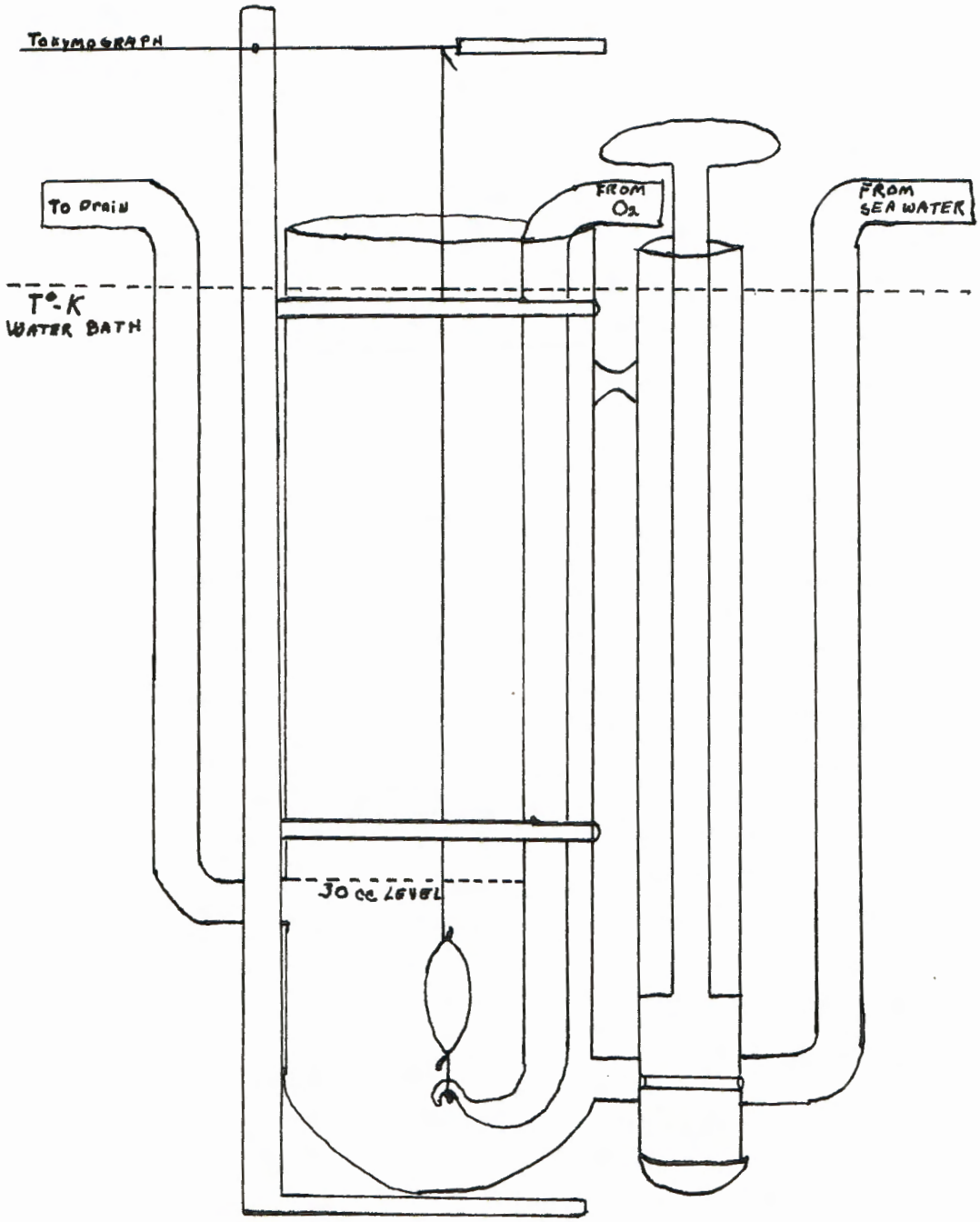


Figure 2. Diagram of the bath containing the Venus heart, set up for an assay procedure.



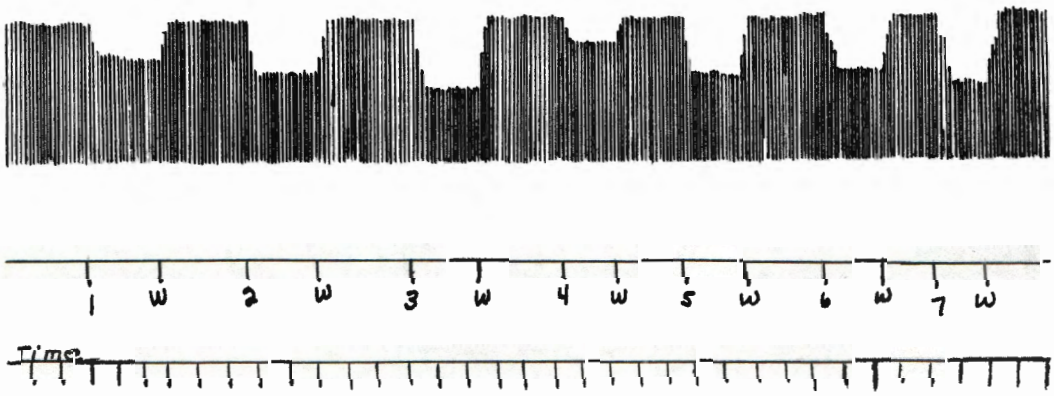


Figure 3. Reproduction of kymograph tracing made by Venus heart during an assay.

1. 0.1 ml. of 1 x 10^{-7} gm./ml. acetylcholine

w - wash 3 times.

2. 0.1 ml. of 1 x 10^{-6} gm./ml. acetylcholine
3. 0.1 ml. of control sample
4. 0.1 ml. of normal muscle sample
5. 0.1 ml. of denervated muscle sample
6. 0.1 ml. of 1 x 10^{-6} gm./ml. acetylcholine
7. 0.1 ml. of control sample

Time - one space is equal to one minute.

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