# TRACER EXPERIMENTS IN CANINE SKELETAL MUSCLE

Thesis for the Degree of M. S.

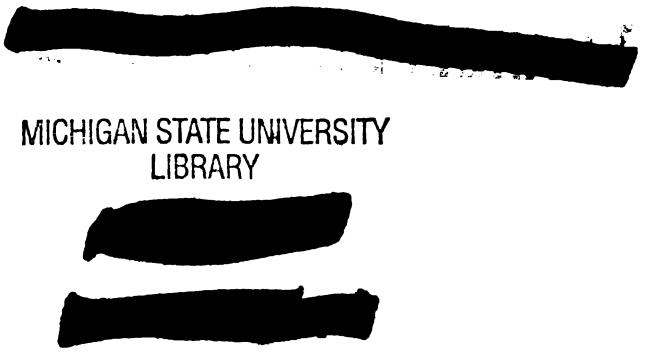
MICHIGAN STATE UNIVERSITY

MICHAEL L. GOODNIGHT

1978







ABSTRACT

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In order to understand more fully the function of the blood vessels in nutritive supply and waste removal, it was desired to investigate experimentally the question of whether blood flow through skeletal muscle occurs in both nutritive and non-nutritive paths.

This study concentrated on precise development of the apparatus and techniques by which to study this problem. In addition, obvious signs of separate parallel blood flow paths were sought for in the resulting data. $C^{14}$ sucrose and $H^{3}$ dextran were injected into the canine gracilis muscle blood supply. Blood samples were collected and the concentration of tracer in the venous outflow measured according to the developed techniques. Although no definite signs of parallel paths were seen, the experiments yielded smooth, consistent data and demonstrated their utility in future experiments of this kind. Further experiments on vascular beds which are known to have shunting pathways, eg. the skin of the canine paw, would be recommended.

# TRACER EXPERIMENTS IN CANINE SKELETAL MUSCLE

By

Michael L. Goodnight

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

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For my parents, Lynn and JoAnn

and my wife, Judith

## ACKNOWLEDGMENTS

The author gratefully acknowledges the assistance of Dr. Donald K. Anderson, Dr. J.B. Scott, the Michigan Heart Association, and his co-worker, Mark Holmes.

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# INTRODUCTION

At present, there is a limited amount of knowledge about transport of solutes in vascular beds. For example, there is a controversy brewing over the mechanism by which vasoconstriction or vasodilation influences transcapillary exchange. There are two major hypotheses proposed to describe the mechanism. First, Renkin (1) has suggested permeability limited transport i.e. permeability of the capillary wall limits the rate of exchange between blood and surrounding tissue. Therefore, as the blood passes through the capillary, equilibrium is not achieved between the blood and the extravascular fluid. Thus, the amount of solute transported to or from the tissue is at least partially dependent on the time available for exchange.

Friedman (2) has advanced the second hypothesis that, as blood perfuses a vascular bed, its flow is in effect split into two parallel streams- nutritive and non-nutritive flow. The nutritive flow stream passes through the capillary bed and achieves equilibrium with the tissue. The non-nutritive flow stream bypasses the capillary bed. Therefore, changes in the amount of solute exchanged with the tissue result from shifts in the flow distribution between both pathways.

To experimentally test their hypotheses on the canine

gracilis muscle, both Renkin and Friedman used radioisotopes of highly exchangeable ions. The former used $K^{42}$ and the latter $Rb^{86}$. However, the question is still unresolved since both investigators have interpreted their results to support their own hypotheses.

In our attempt to study this problem, we have used techniques developed for the analysis of flow distribution in chemical reactors. Levenspiel (3) has shown that pulse injection studies (similar to the studies done by Renkin and Friedman) can, if done properly, yield information on the flow patterns in the catalyst bed of a chemical reactor. For instance, it is possible to detect the presence of parallel streams with different residence times (channelling) or dead space in the bed.

We have attempted to apply these same methods to flow in a vascular bed. The method consists of the following procedures:

1) A non-diffusible tracer, $H^3$ labeled dextran, is injected as a bolus into the blood stream perfusing a vascular bed. Serial samples of the venous outflow are taken during the time required for all of the tracer to exit the bed. Using radiotracer counting techniques, the concentration of tracer in each sample is determined.

2) A diffusible tracer is also injected in the same bolus in order to test for nutritive versus non-nutritive flow. If the tracer in one or both of the parallel streams (providing they exist) can exchange with surrounding tissue, we would expect a delayed washout of tracer from the muscle. One

stream would show this delay while the second parallel stream would not if Friedman's hypothesis is correct.

3) The resulting concentration versus time data are plotted and inspected for obvious signs of parallel paths which would show as two peaks in the curve (3).

4) Electrical stimulation of the muscle was conducted in the experiments to determine the effects, if any, of exercise on the vascular flow and the resulting tracer curves.

In addition, several experiments also involved a histamine infusion prior to tracer injection to view its effect on tracer recovery.

$C^{14}$ tagged sucrose was used as this will pass through the capillary wall (though not as easily as $Rb^{86}$) but will not enter the cells. This permits back-diffusion of extracted tracer into the blood and allows recovery of a larger fraction of the injected tracer.

A diffusible and non-diffusible tracer were used in these experiments in anticipation of a need for data concerning diffusible and non-diffusible tracer washout curves such as would prove useful in an analysis conducted along the lines developed by Borghi (4).

## EXPERIMENTAL METHODS

### The Choice of Tracers

In accordance with the diffusible models (4), a tracer was needed which would cross capillary membranes and diffuse into the interstitial fluids, but would not be taken up and retained by the tissue cells. It was important that this tracer not be taken up by the cells to enhance the process of diffusion back into the capillary flow stream and insure detection.

The tracer selected for this purpose was $C^{14}$ labeled sucrose. Sucrose readily crosses capillary membranes, distributing itself in the extracellular space of the tissue, but is not taken up by the cells. It is for this reason, in fact, that sucrose is commonly used for measuring the extracellular fluid volume of the body (5,6).

In accordance with the non-diffusible models (4), a tracer was required which would not cross the capillary membranes (referred to as the non-diffusible tracer).

$H^3$ labeled dextran was selected for this purpose. Dextran is a polysaccharide of molecular weight of 60,000-90,000. This was chosen over $I^{131}$ labeled albumin, which was originally suggested, because tritium has a much longer half life than $I^{131}$ ($t^{\frac{1}{2}}$ of $I^{131}$ is 8.03 days) and could be considered to have a constant activity. Tritium labeled dextran may be differentiated from $C^{14}$ labeled sucrose in liquid scintillation counting (7), thus both the diffusible and the non-diffusible tracers may be injected at the same time. As for the

permeability of the capillary membranes to 60,000-90,000 MW dextran, studies have shown that it is less permeable than blood proteins (8). The major excretory pathways of dextran in the body are the liver, kidneys, and the gastrointestinal tract (9).

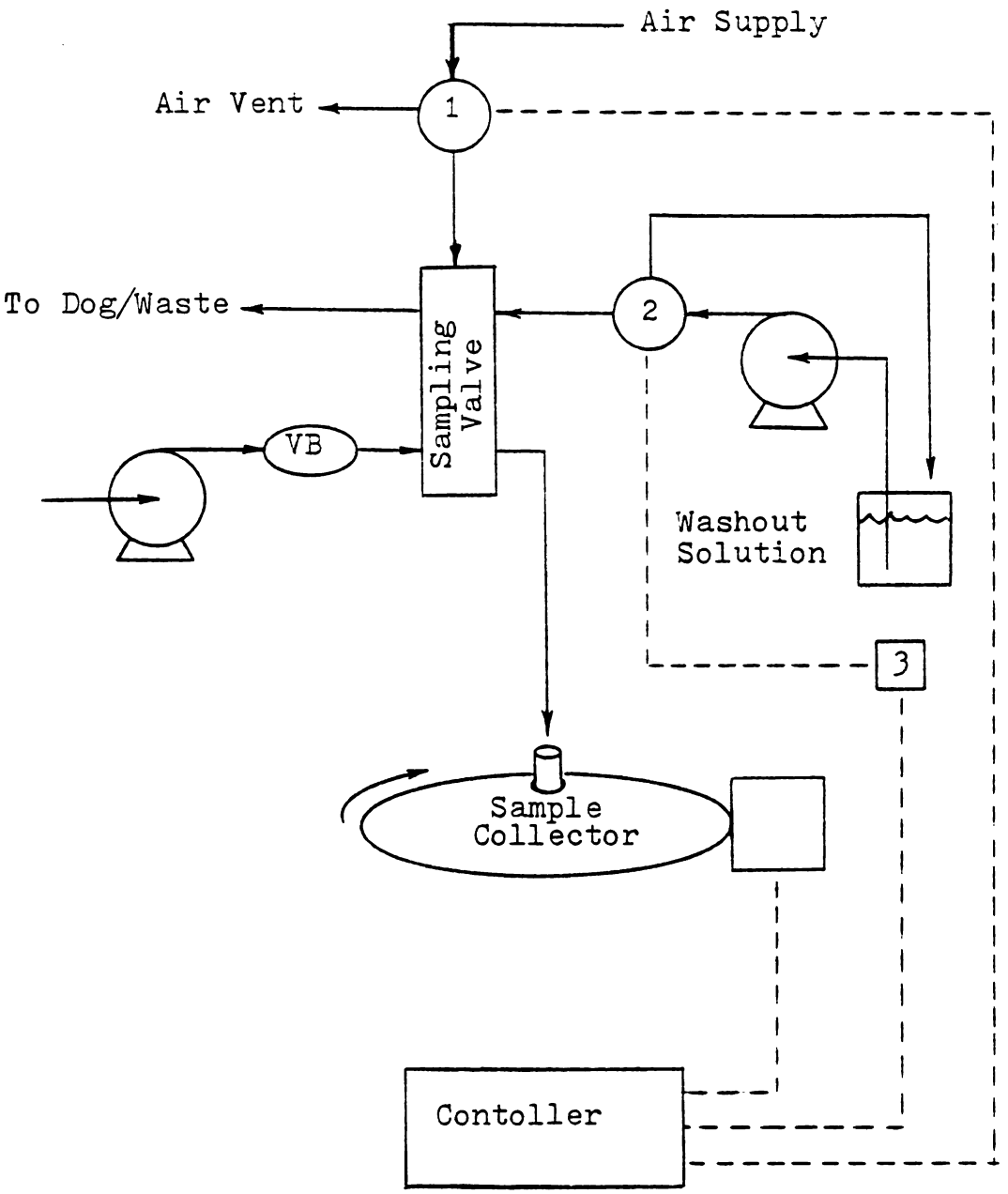
For those experiments in which $C^{14}$ sucrose alone was used, the tracer solution consisted of 3.0 $\mu$Ci/cc in 0.85% normal saline. For the dual tracer experiments, the solution was 1.25 $\mu$Ci/cc of $C^{14}$ sucrose and 23.33 $\mu$Ci/cc of $H^{3}$ dextran, also in 0.85% normal saline. Injection volumes were 0.25 cc with $C^{14}$ sucrose alone or 0.5 cc with the dual tracer.

Experimental Layout

Figure 1 shows a schematic of the experimental setup used. The vascular bed was pump perfused by blood from the femoral artery. Venous blood passed through the sampling valve and the portion not collected was discarded when the experiment was in progress. Washout solution was supplied by a constant flow pump.

A solenoid valve (No. 2) directed the flow of the washout solution either back to the solution reservoir or to the sampling valve to flush the sample loop into collection vials which were serially positioned by an automatic fraction collector.

A three way solenoid valve was used to direct a supply of high pressure air to the sampling valve for activation in order to shift the internal flow pattern of the sampling valve.




VB - Vascular bed being perfused
1 - Three way valve to direct air to sampling valve
2 - Three way valve to direct washout solution
3 - Delay relay
Washout solution consists of; 1200 ml of 0.85% saline solution and 4 ml of sodium heparin (5000 USP units/ml)
---------- Control connections

Figure 1. Experimental Setup

Finally, the controller is shown with its control pathways shown as dashed lines. More about the controller will be given in the section marked Electronic Controls.

The Sampling Valve

The heart of the experimental apparatus was the sampling valve used to take intermittent blood samples from the venous side of the vascular bed being perfused. A valve was needed which would collect blood samples of a constant size for scintillation counting, while not interfering with the normal flow of blood through the vascular system. The Altex Scientific Inc. Model no. 201-63 stream sampling valve chosen had the advantage of having little or no dead space and provided samples of reproducible volume. The flow pathways of this valve were enlarged to 1/16 inch to reduce the flow resistance through the valve. The flow pattern through the valve (refer to Figure 2) was determined by the position of an internal slide which was pneumatically activated and spring returned.

In the filling position, the blood from the dog flowed into the valve, through the collection loop, out of the valve, and was either returned to the dog or discarded. Meanwhile, the flow path from the washout solution was connected to a return path to the dog (however, there was no flow of washout fluid at that time). The path to the collection vials was connected to a dead end during this filling period.

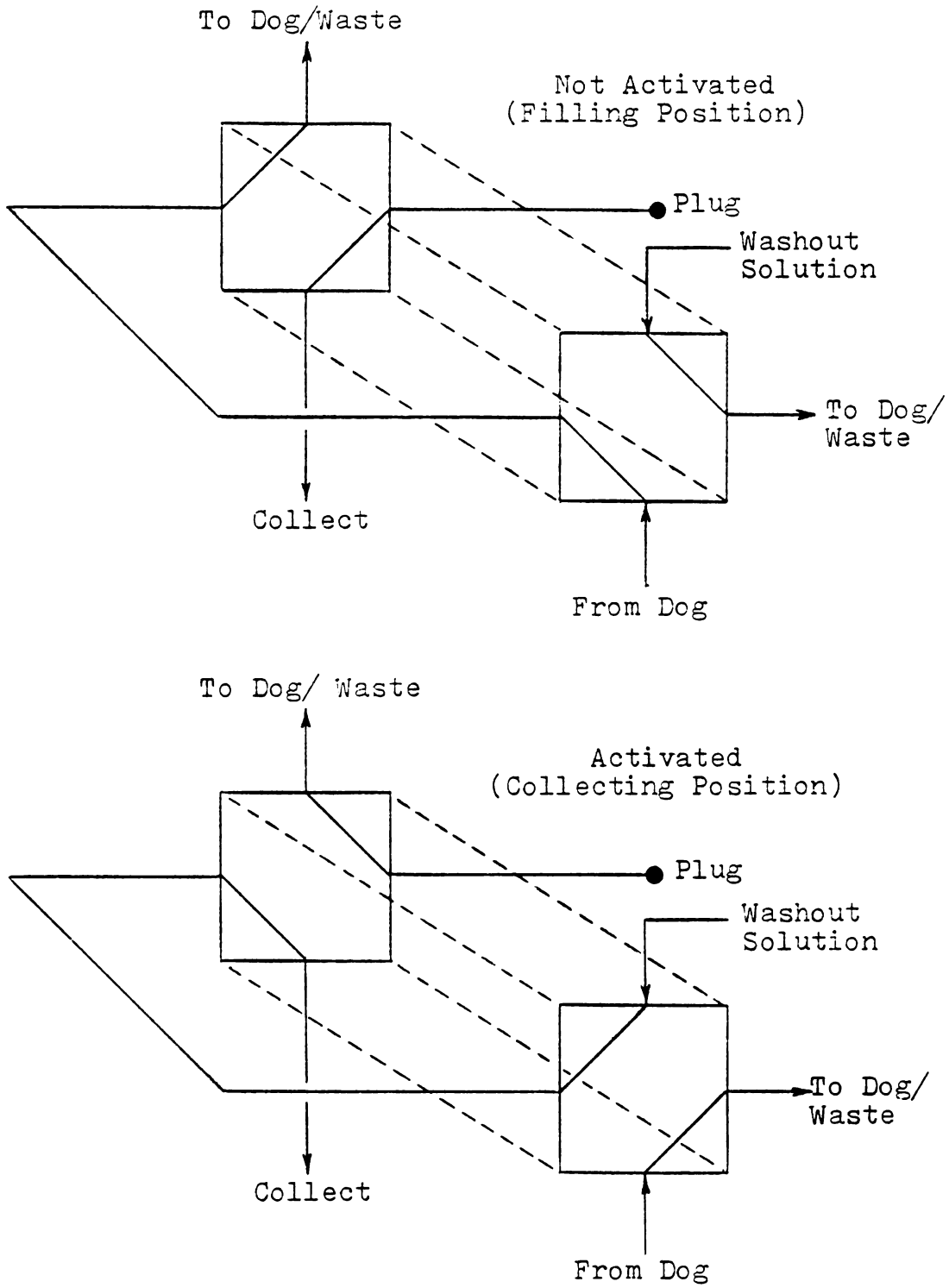



Figure 2. Valve Configuration

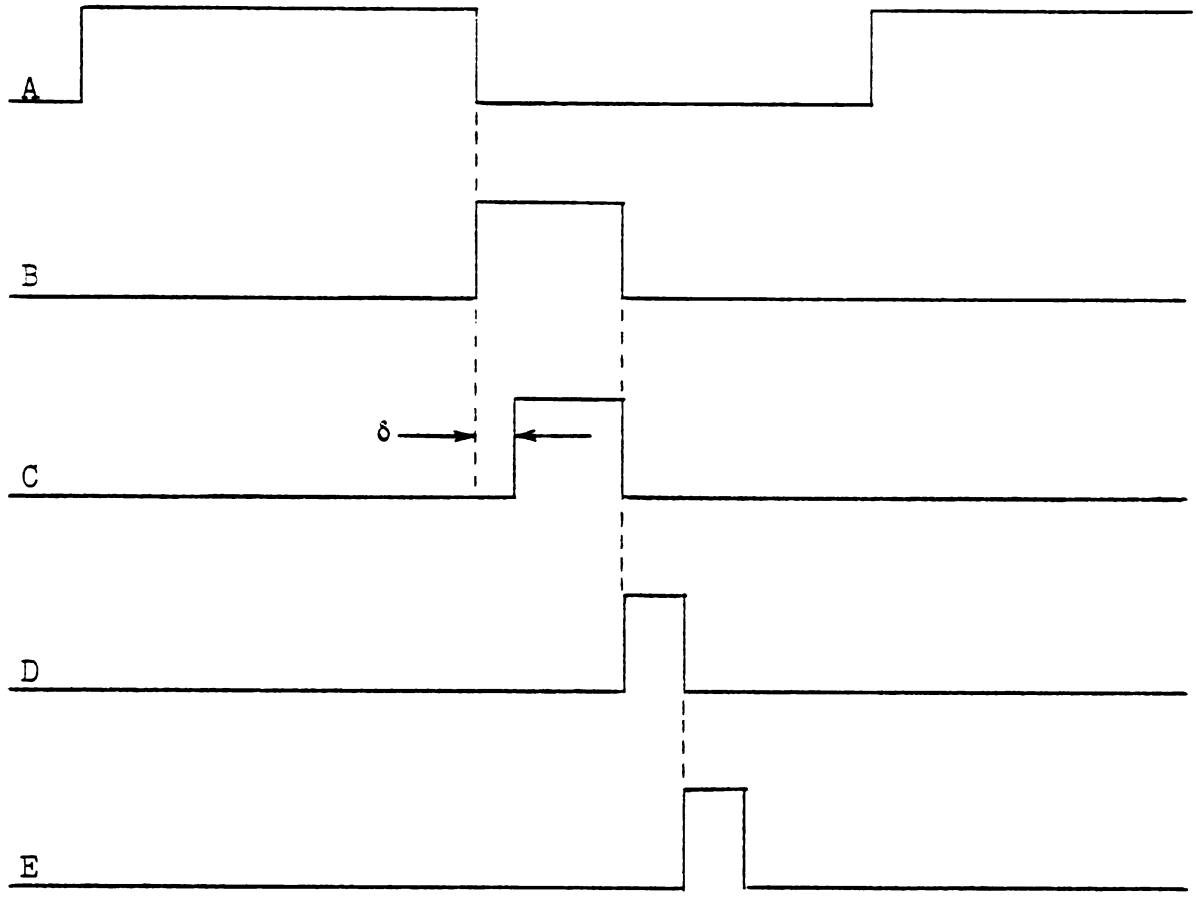
Upon pneumatic activation by a high pressure air source directed by a three-way valve, the internal flow pattern was changed to the sampling position (see Figure 2). In this position, the sample loop was washed out with solution and collected in a sample vial. During this period, the blood from the dog went either back to the dog or to waste, depending upon whether samples were being taken at that time or not.

The volume of the sample loop was determined by passing a solution of hydrochloric acid of a known concentration through the loop, washing out the loop with distilled water into a sample vial, and then titrating several such samples with a standard sodium hydroxide solution. By this method, the loop volume was determined to be 0.2464 ± 0.00079 ml (95% level, n=25).

Electronic Controls

To obtain samples at regular time intervals, an electronic controller was constructed. This control unit is responsible for activation of the sampling valve from the filling to the collecting position, for activation of the solenoid to direct washout fluid through the sampling valve, and for advancement of the fraction collector to position a new vial beneath the sampling valve.

Figure 3 shows the various signals generated in the control unit. Signal A is a variable time base which is used to set the overall time span between samples. As this signal




A - Time base
B - Sampling valve activation
C - Washout valve activation
D - Delay
E - Fraction collector activation
δ - Delay between valve activations

Figure 3. Controlling Signals

falls off, the pulse B is generated activating the sampling valve from the filling to the collecting position.

The fall of the time base signal A also activates a delay relay which, after a short time to allow complete positioning of the sampling valve, activates signal C to start the flow of washout fluid through the sampling valve.

Upon the cessation of signals B and C, a delay signal (D) is activated to insure that any drop of liquid remaining on the tubing above the sample vial has a chance to drip into that vial before a new one is moved into position.

The fall in D produces signal E causing the fraction collector to advance.

The duration of each of these signals was adjustable with the time base having a range up to 30 seconds in length.

Experimental Preparation

Mongrel dogs (16-20 kg.) were anesthetized with 18 to 20 cc of a sodium pentobarbital (Nembutal) solution (50 mg/cc) and respirated mechanically (Harvard Apparatus dual phase control respirator, 15 rpm, 250 cc/stroke, output ratio=45).

With the gracilis muscle exposed, all blood vessels communicating with the muscle except the major artery and vein and those entering at the origin and insertion were ligated. The gracilis artery was connected to a pump to be perfused at constant flow (using blood from the femoral artery) and the venous outflow directed to the sampling valve. Blood from the normally open path of the valve was returned to the

femoral vein. In addition, all nerves to the muscle were cut and the obturator nerve leading to the gracilis muscle was attached to an electrical stimulator.

The washout solution consisted of a 0.85% saline solution with 4 ml of 5000 USP units/ml sodium heparin solution added per 1200 ml of saline solution. This was circulated by the washout pump through the directional valve (see Figure 1) and back to the supply reservoir.

0.2 ml of a 60 wt % perchloric acid solution was placed in each sample vial. This solution, along with a 30 wt % hydrogen peroxide solution added later, was used to bleach and solubilize the blood to reduce interference during liquid scintillation counting. Perchloric acid-tracer standard solution (0.2 ml) was added to determine counting efficiencies of the $H^3$ dextran and $C^{14}$ sucrose tracers used. This is discussed in more detail in the analysis of samples section later on.

Statham pressure recording transducers (Model no. P23Gb) were used to monitor and record onto a strip chart the arterial pressure, perfusion pressure, and venous pressure of the dog. An indexing signal (actually, the sampling valve pulse) from the controller was also recorded to provide a record of the time interval between samples.

In the later experiments, when both $C^{14}$ sucrose and $H^3$ dextran were injected, the blood was discarded during sampling to prevent recirculated tracer from being collected. Dextran (6% w/v) in 0.9% saline aws used to replace this

lost volume during the experiment.

The flow rate to the muscle was adjusted to give a perfusion pressure approximately equal to systemic pressure.

## Experimental Procedure

Several blanks and standard samples were obtained before introduction of the tracer. Then, the tracer was injected at a point close to the muscle, while the time was noted on the strip chart for future reference. At that time, the blood was diverted to a waste container and the (nonradioactive) dextran infusion was begun.

Between 50 and 60 samples (plus standards and blanks) were collected for each experiment. For the "exercise" experiments, electrical stimulation was used on the obturator nerve (6Hz @ 6 volt, 1.6 msec duration) for 30 seconds immediately prior to the tracer injection.

In some cases a third run was made during infusion of a histamine solution (10 g/cc @ 1.23 cc/min). The infusion was started approximately 5 minutes prior to the injection.

After each run, the blood was directed back to the dog to prevent excessive loss, the dextran infusion stopped, and the muscle allowed to recuperate from the exercise.

## Analysis of Samples

The determination of the amount of tracer(s) present in each sample was accomplished by means of liquid scintillation counting. Detailed explanation of the general counting principles will not be given here due to the amount of time

needed to cover the material adequately. There are many excellent sources on the subject readily available (7,10,11).

To reduce the amount of quenching due to the highly colored blood samples, a bleaching procedure was employed as outlined in a Beckman Instruments Co. publication (11). Briefly, this involved the addition of 60% $HClO_4$ (0.4 ml/1.0 ml blood) and 30% $H_2O_2$ (0.8 ml/1.0 ml blood) as bleaching and solubilizing agents. After the samples were bleached for 3-4 hours, 3-4 drops of 15% fresh ascorbic acid solution (per 1.0 ml blood) were added to act as oxygen scavengers in order to alleviate chemiluminescence. Then, 10 ml of Beckman ReadySolve HP scintillation cocktail were added and the samples shaken. The samples were ready to count after the solid matter was allowed to settle.

Standardized samples were used to determine the counting efficiency for each set of samples. The standards consisted of preparations of known concentrations of tracers mixed separately in a perchloric acid solution (one of the bleaching agents). This solution was then used in place of the normal $HClO_4$ solution for 10 samples. The counting efficiency was the same for the standard and the regular samples since the standard solution mixed with the unlabeled blood before the injection in the same way that the pure $HClO_4$ solution mixed with the labeled blood. Thus, the amount of tracer in the labeled blood samples was calculated from the net CPM (counts per minute) of a given sample divided by the average net CPM per ml tracer in the standard samples. This was done

for both $C^{14}$ and $H^3/C^{14}$ injections because the $C^{14}$ and $H^3$ counts could be separated onto different channels and compared to either set of standards.

All samples were counted on a Nuclear Chicago liquid scintillation machine (Model no. 6860 Mark I) for 20 minutes each. For that length of time per sample, the percent error in the counting is only 4.4% for CPM as low as 100 (95% level of confidence).

After the ml of tracer in each sample was determined, a value E was calculated for each. This value E was defined by:

$$E = \frac{f(t)\ v}{V} \quad (sec^{-1})$$

where: $f(t)$ = fraction of the total injected tracer which appeared in the sample collected at time t.

or $f(t) = \frac{\text{ml tracer in sample collected at time t}}{\text{ml tracer injected}}$

$v$ = blood flow in ml/sec

$V$ = volume of blood sample = sample loop volume.

This value E is the same as the E discussed in Levenspiel (3). Levenspiel defines the fraction of the exit stream of age between t and t+dt as Edt. If t is defined as the time when the first bit of our sample enters the sample loop and t+dt as the time when the sample is collected, then Edt is equivalent to the term f(t) the fraction of the total tracer which is in the sample. And dt, the time from when the sample first starts to enter the sample loop to when the sample is collected (which is a small time interval), may be written as V/v, where V is the sample loop volume and v is the

volumetric flow rate through the sample loop. Then

$$Edt = \frac{E\,V}{v} = f(t) \qquad \text{or} \qquad E = \frac{f(t)\,v}{V}$$

as stated before.

Levenspiel also states that $\int_0^\infty Edt$ should equal 1. Table 1 shows the tracer "recoveries" for the various experiments. These "recoveries" were calculated from a numerical integration of the E vs. time data over the length of the experiment. These values give an idea as to how much of the tracers exited the vascular bed during the experiment.

Table 1. Tracer Recoveries

| Figure | | $C^{14}$ | $H^3$ |
|---|---|---|---|
| 4 | □ | 0.7035395 | - |
| 5 | □ | 0.7148130 | - |
| 6 | □ | 0.9496238 | - |
| 6 | ○ | 0.9216082 | - |
| 7 | □ | 0.8251231 | - |
| 7 | ○ | 0.6812981 | - |
| 8 | □ | 0.7952803 | - |
| 8 | ○ | 0.8097591 | - |
| 9 | □ | 0.9460958 | - |
| 9 | ○ | 0.9714919 | - |
| 10 | □ | 0.9270879 | - |
| 10 | ○ | 0.9852182 | - |
| 11a | | 0.8547825 | 0.9855676 |
| 11b | | 0.8469506 | 0.9636616 |
| 12a | | 0.9098015 | 0.8880417 |
| 12b | | 0.7162707 | 0.6958571 |
| 12c | | 0.8354927 | 0.5238641 |
| 13a | | 0.9054458 | 0.8619089 |
| 13b | | 0.7465478 | 0.4401488 |
| 13c | | 0.6588887 | 0.4171850 |
| 14a | | 0.8087099 | 0.7340553 |
| 14b | | 0.8376521 | 0.5925857 |
| 14c | | 0.7493408 | 0.4821045 |

## RESULTS

The following graphs, Figures 4 through 14c, show the results of the experiments employing the equipment and the methods outlined.

Figures 4 and 5 show the E vs. time curves following injection of $C^{14}$ sucrose and no stimulation of the muscle. Each figure represents an experiment performed on a particular muscle specimen.

Figures 6 - 10 show the E vs. time curves following injection of $C^{14}$ sucrose both before and after electrical stimulation of the muscle (see page 13). Each figure represents a set ofexperiments performed on a particular muscle specimen.

Figures 11 a.b show the E vs. time curves for $H^3$ dextran and $C^{14}$ sucrose after both were injected simultaneously. Note that for each sample an E value was calculated for $C^{14}$ sucrose as well as for $H^3$ dextran and both plotted versus time. Figure 11a shows the results prior to stimulation while 11b shows the results after electrical stimulation.

Figures 12a,b,c, 13a,b,c, and 14a,b,c show the E vs. time curves for $H^3$ dextran and $C^{14}$ sucrose after simultaneous injection. The figures marked 'a' show the results before stimulation; those marked 'b' show the results after stimulation; and those marked 'c' show the results after histamine infusion (see page 13). Each set of figures a,b,c, represents a set of experiments performed on a particular muscle specimen.

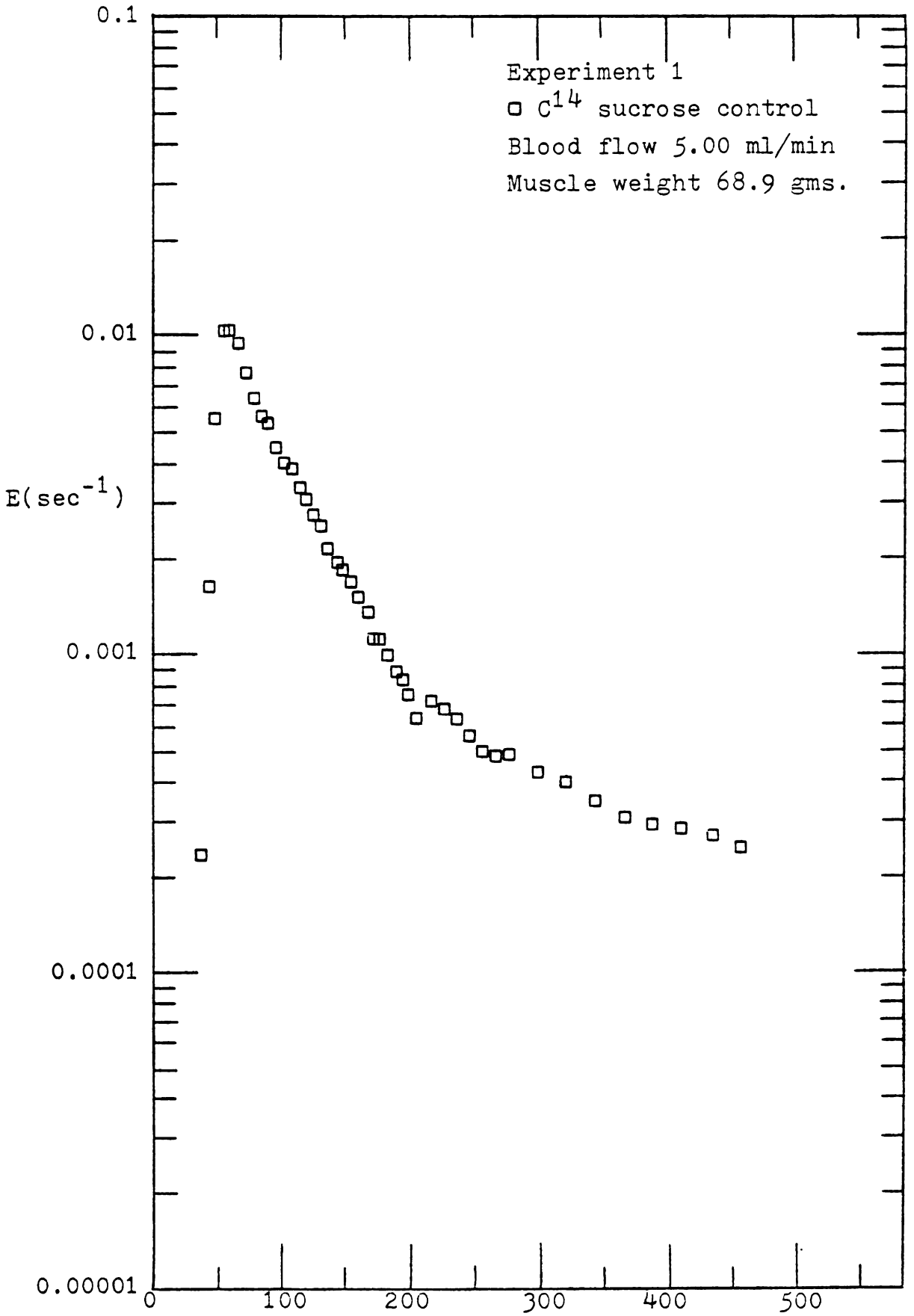



Figure 4. Expt. 1

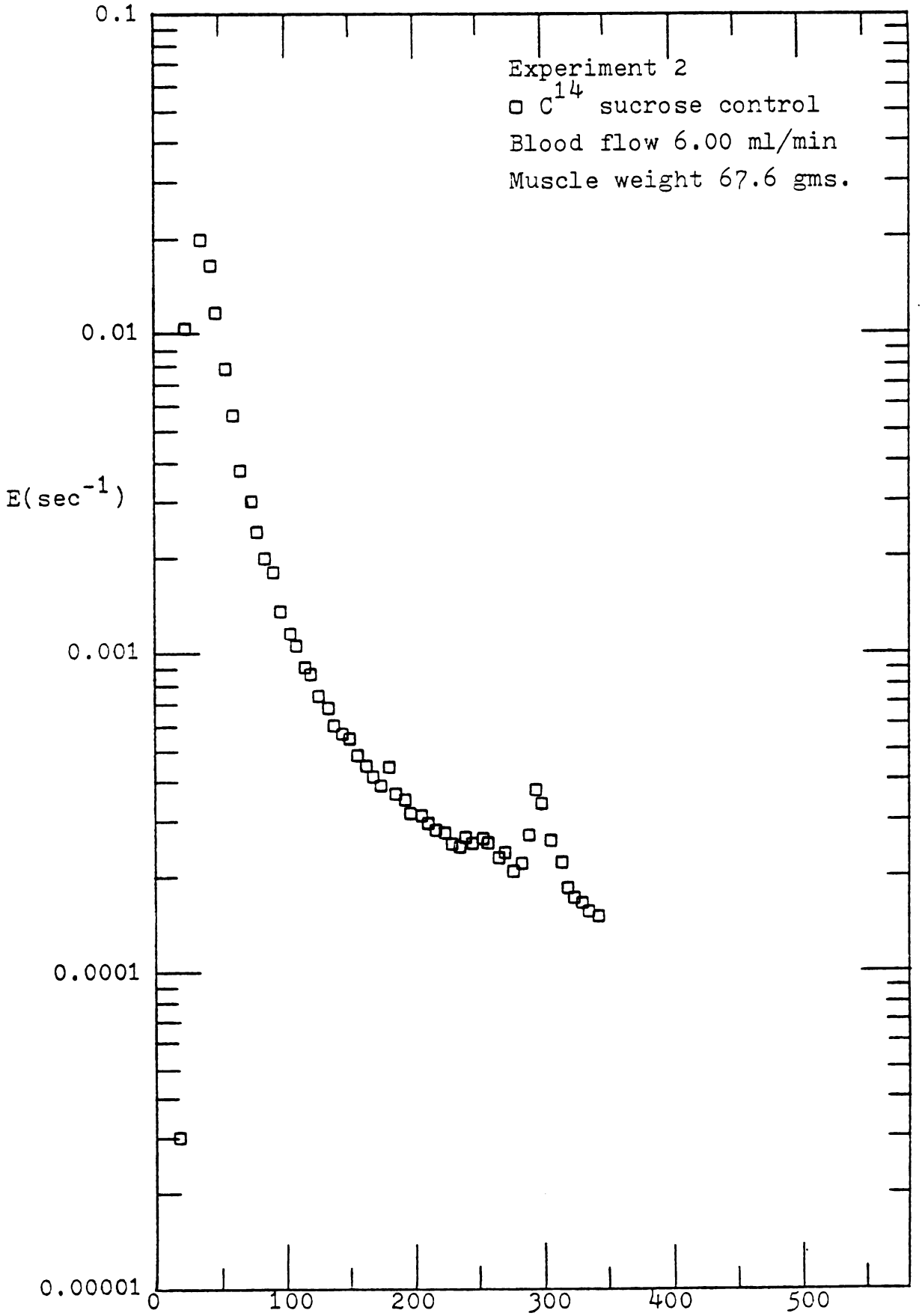



Figure 5. Expt. 2

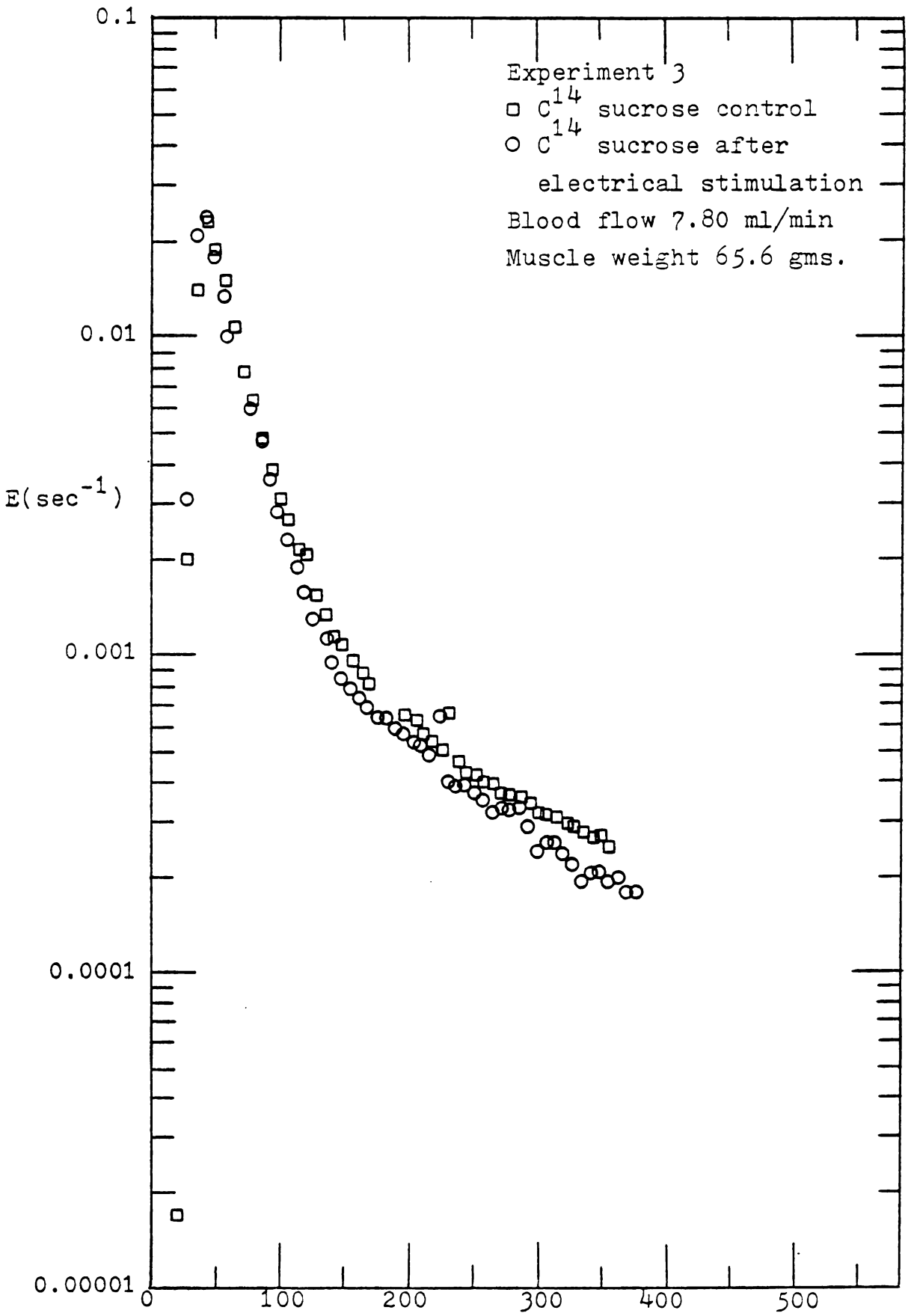



Figure 6. Expt. 3

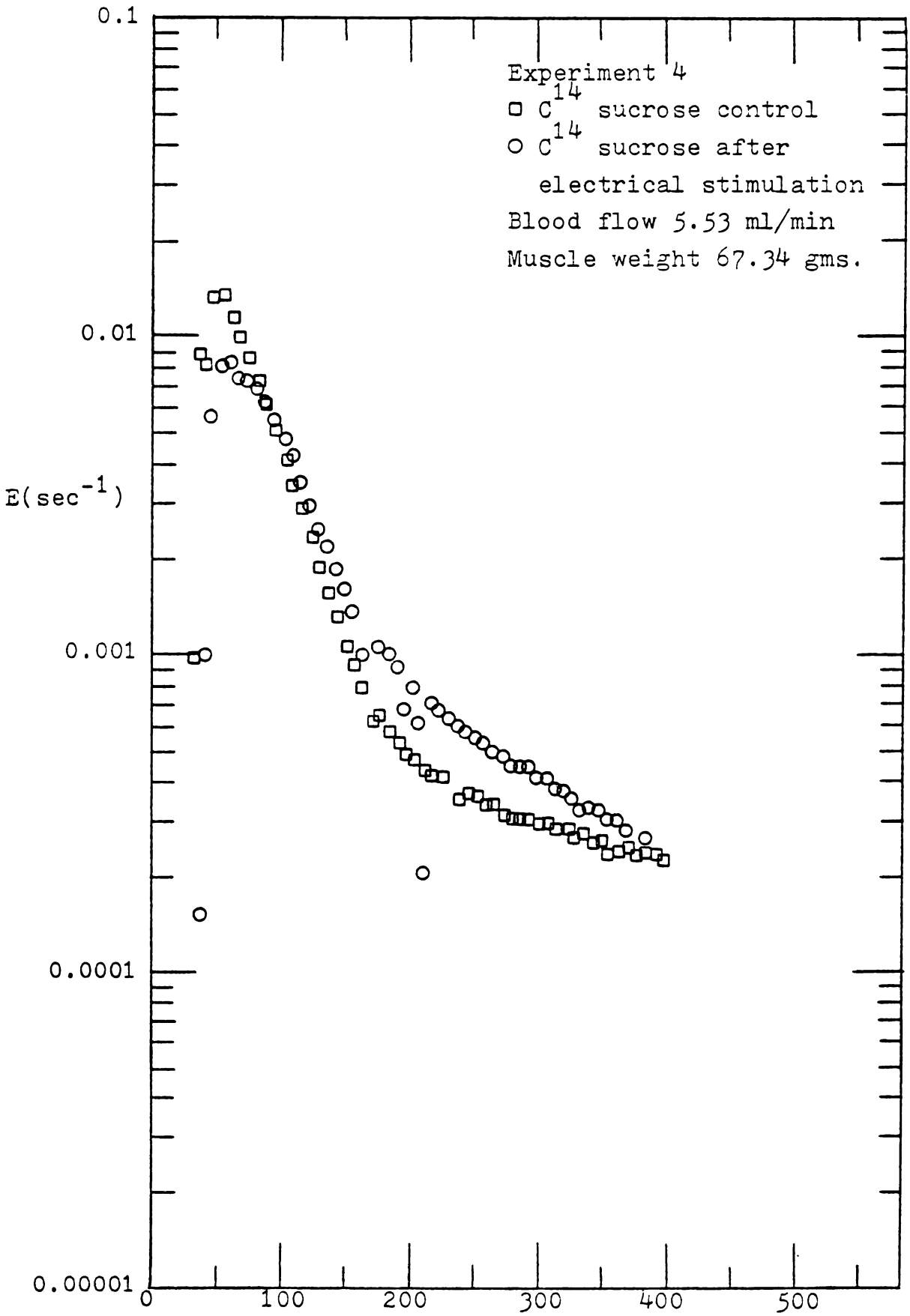



Figure 7. Expt. 4

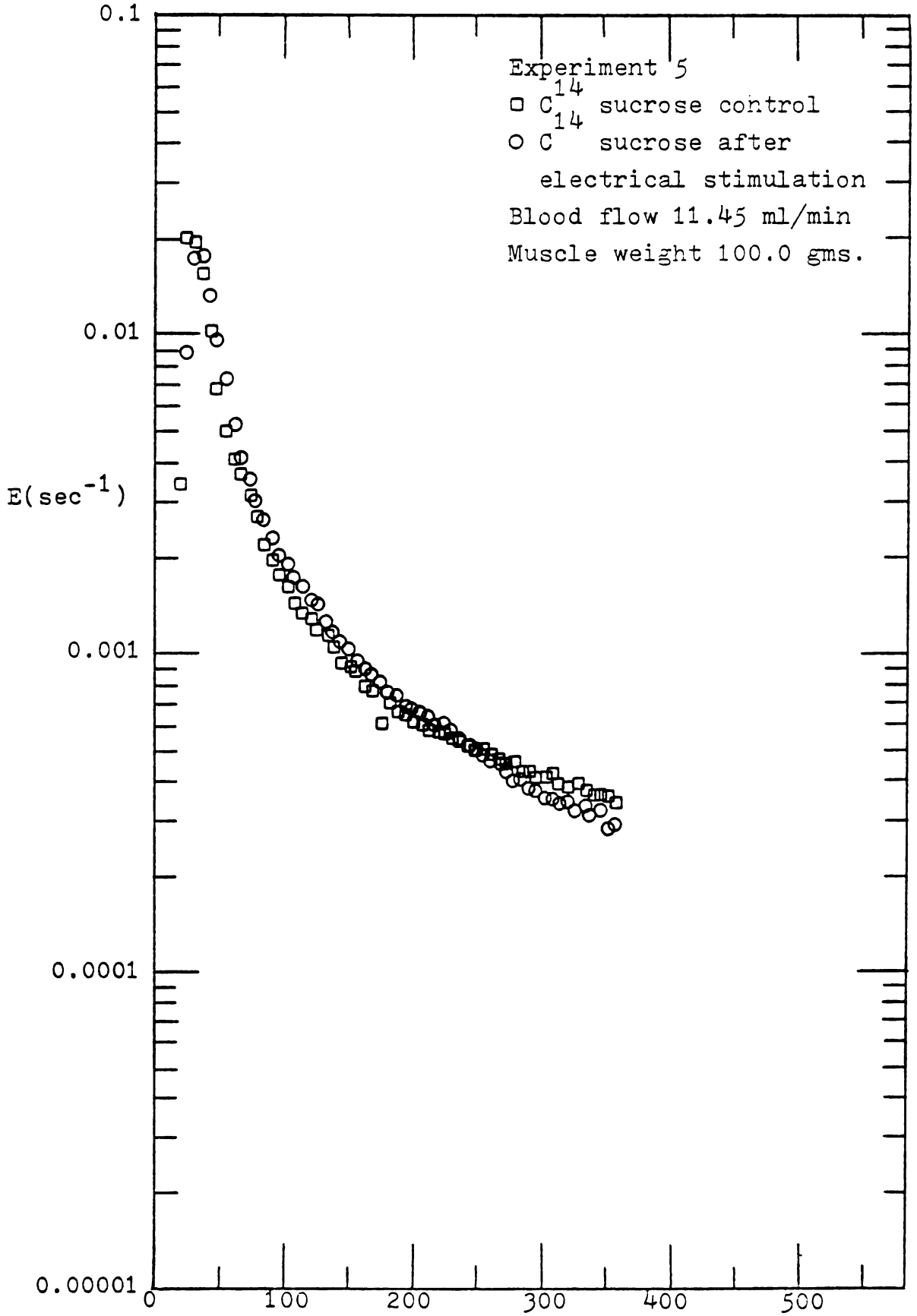



Figure 8. Expt.5

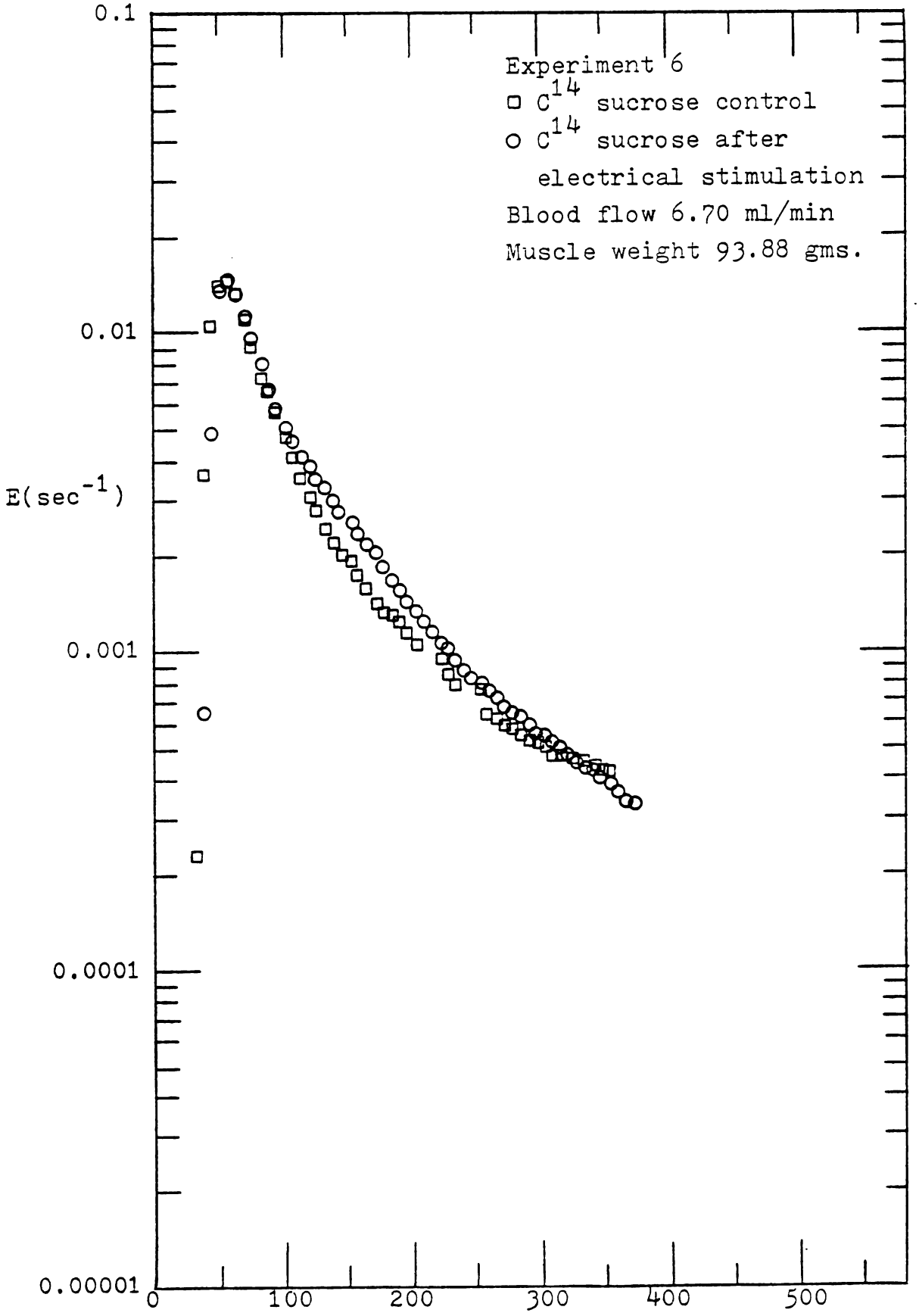



Figure 9. Expt.6

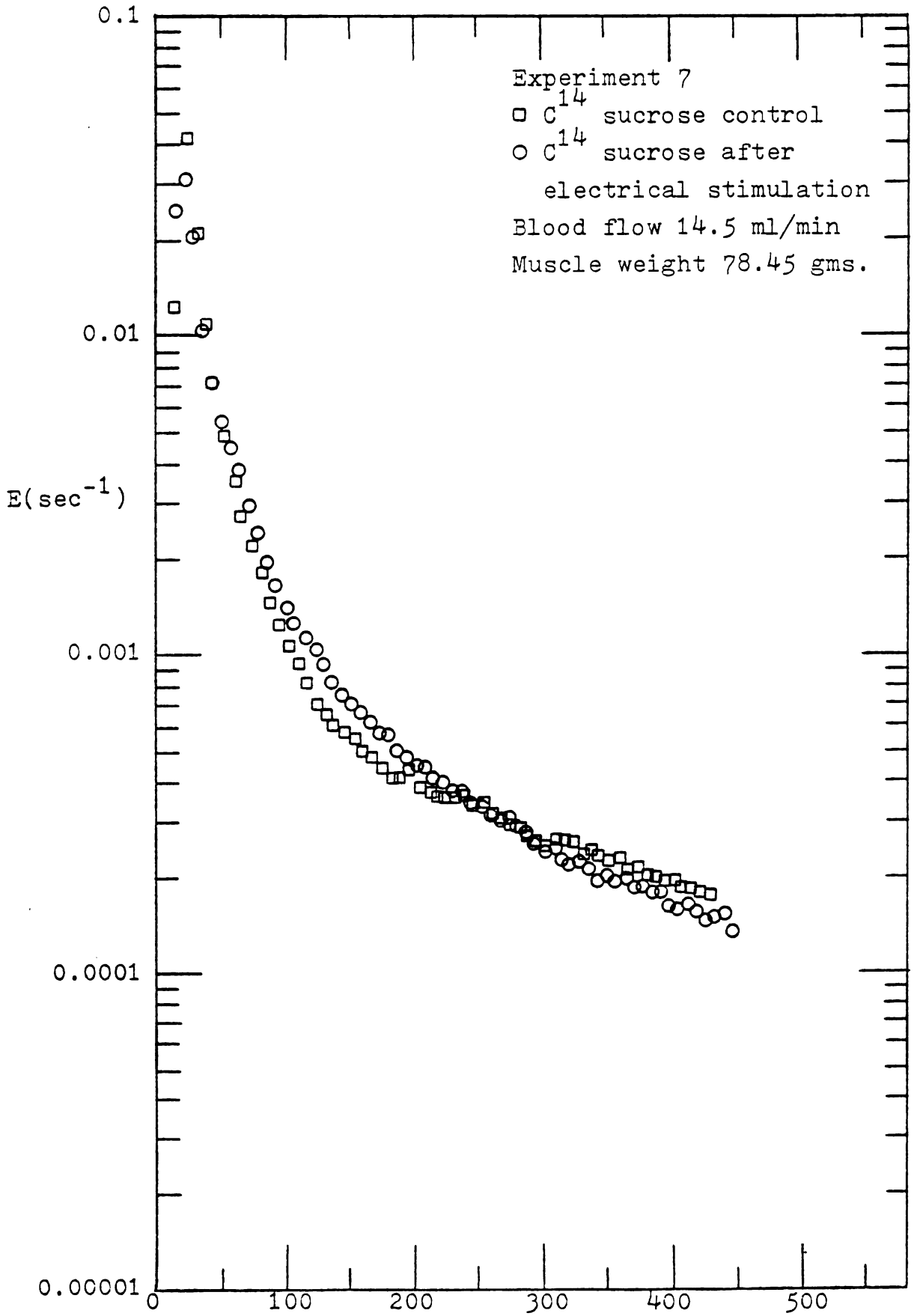



Figure 10. Expt. 7

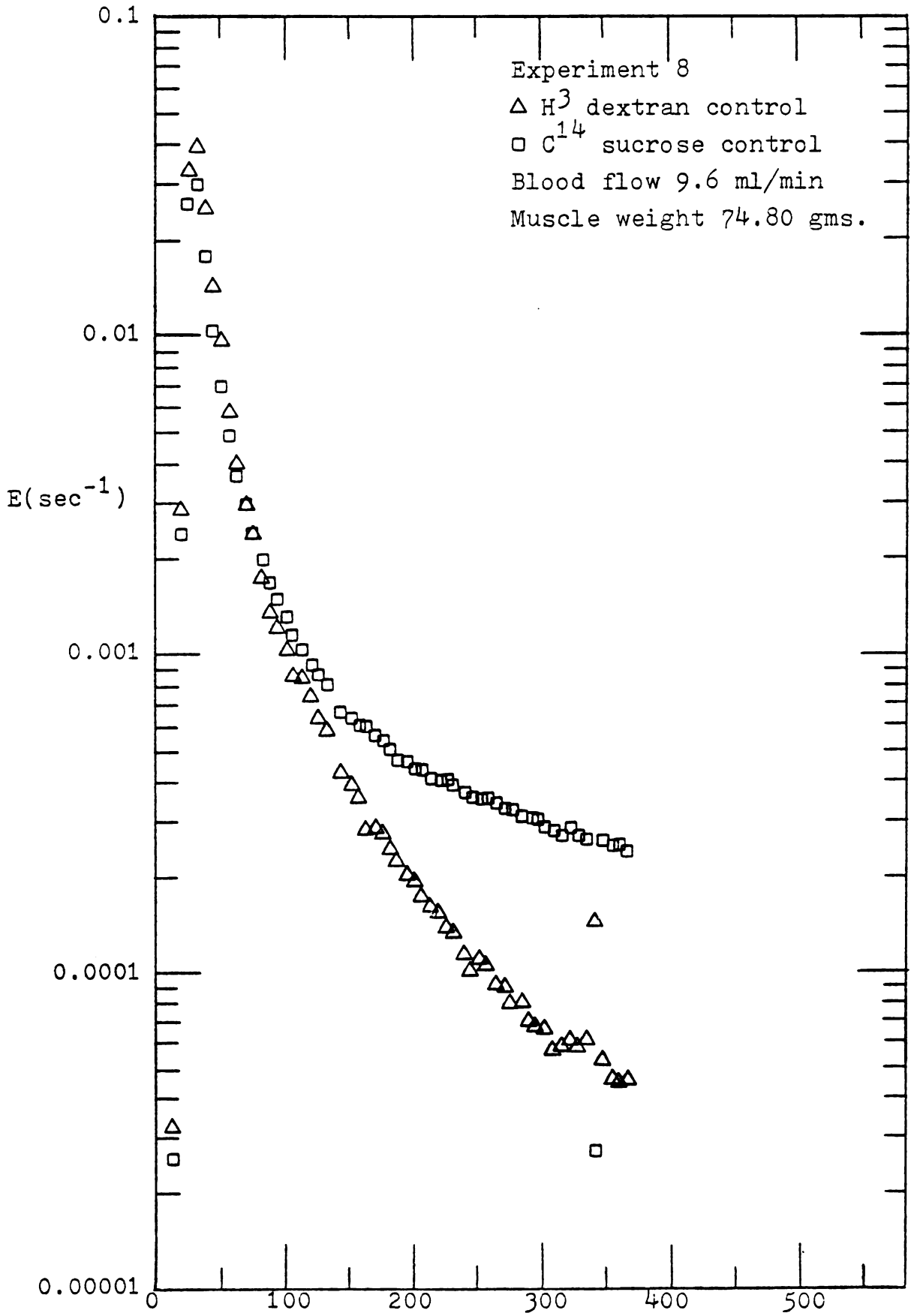



Figure 11a. Expt. 8

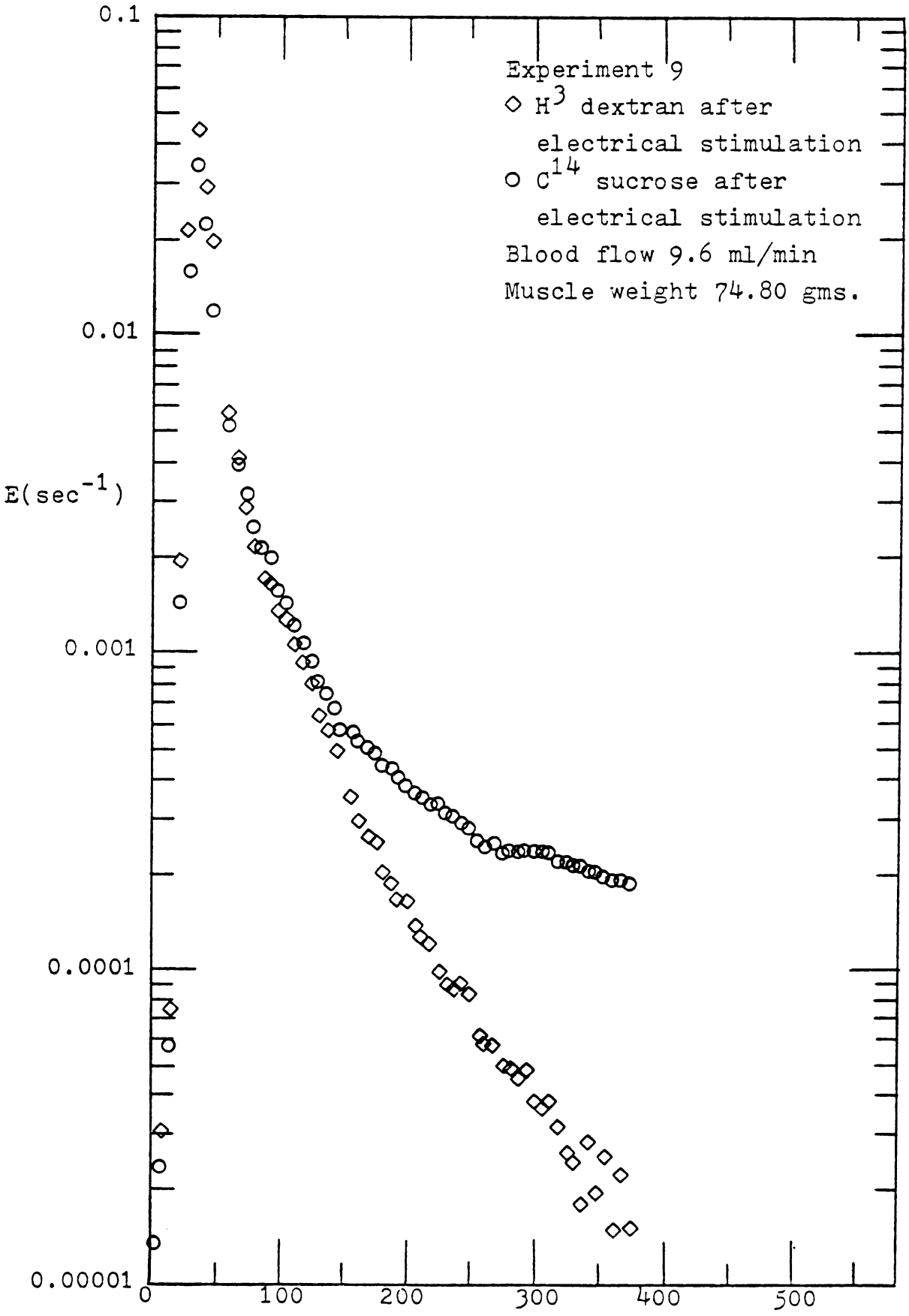



Figure 11b. Expt. 9

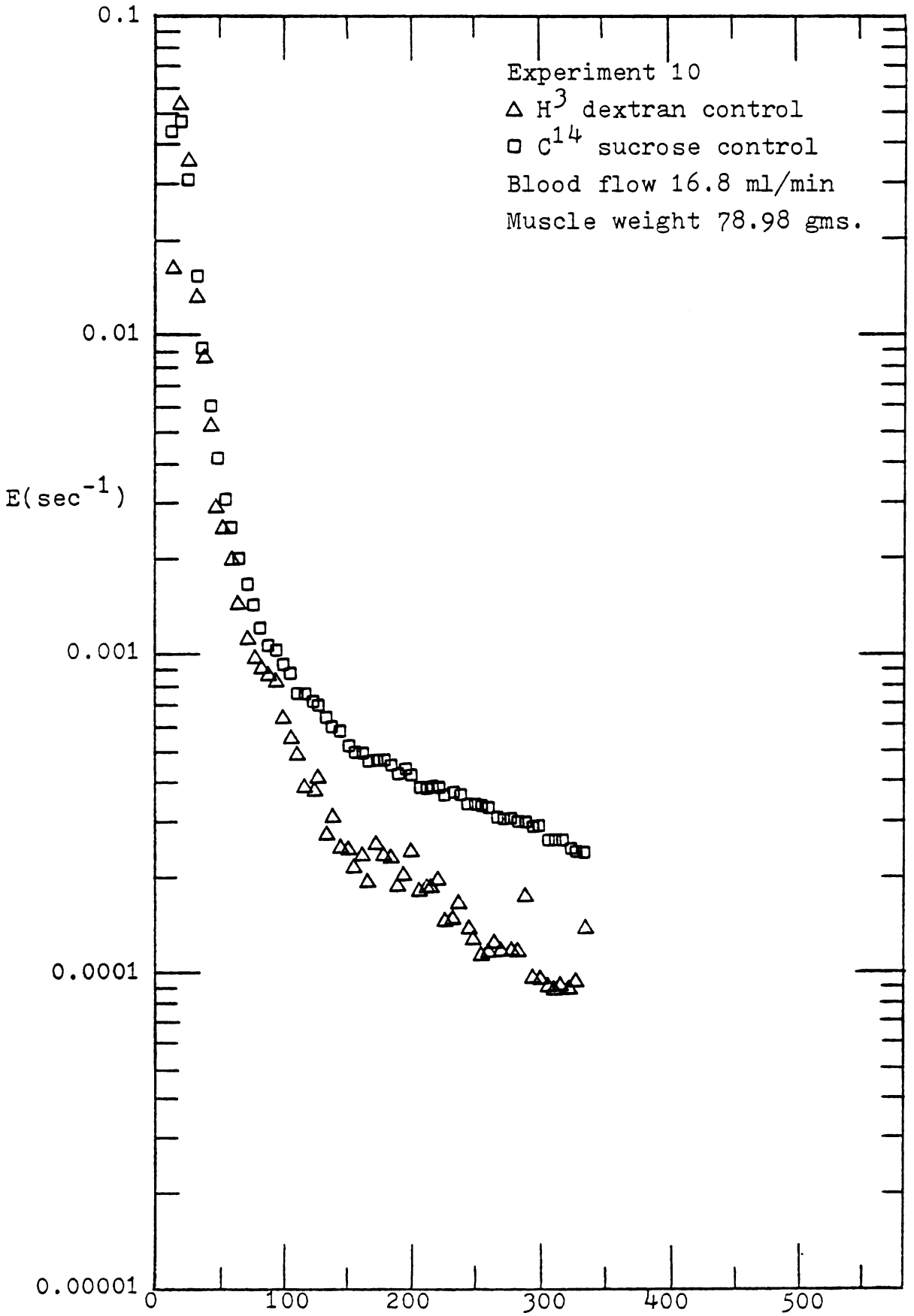



Figure 12a. Expt.10

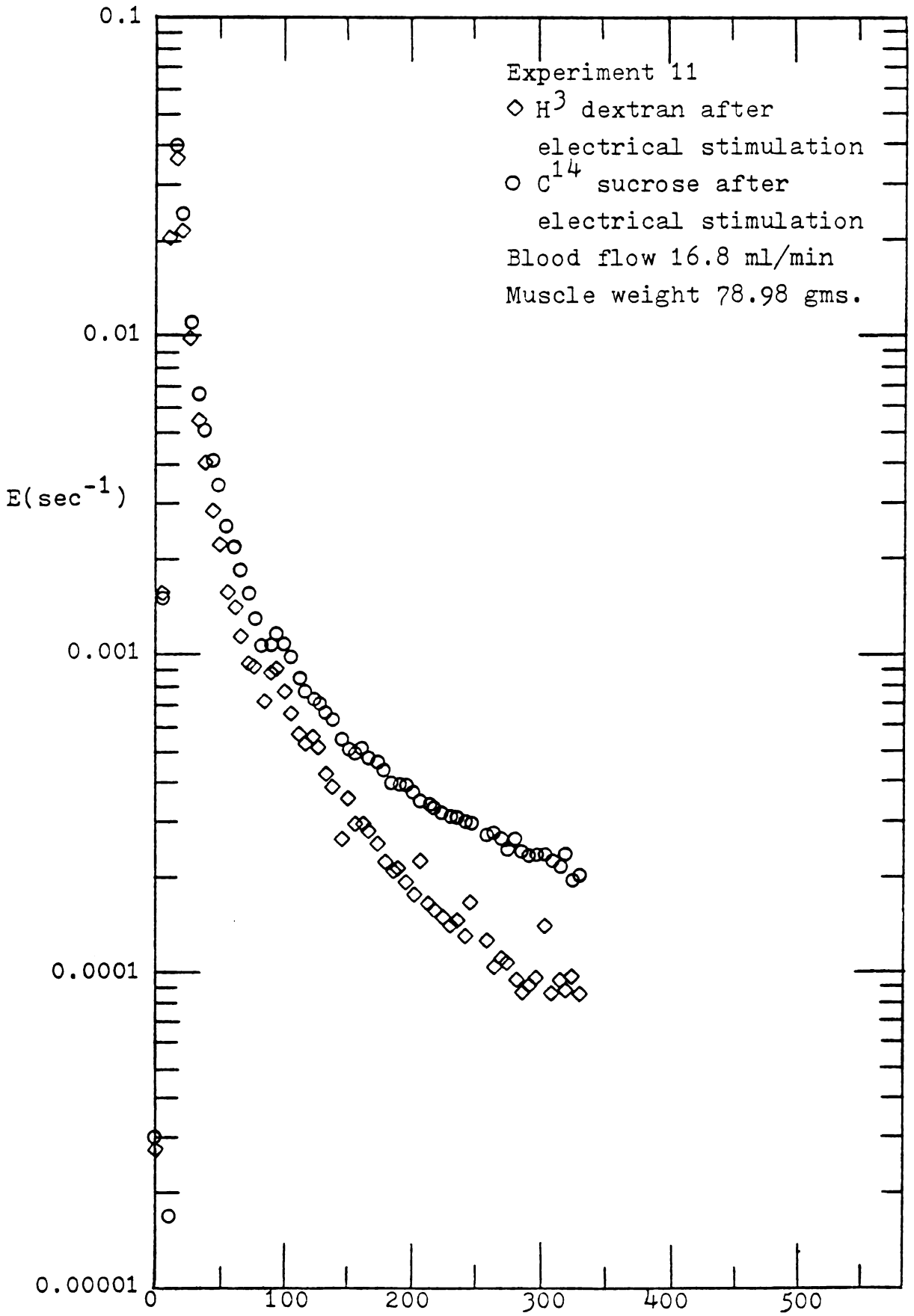



Figure 12b. Expt. 11

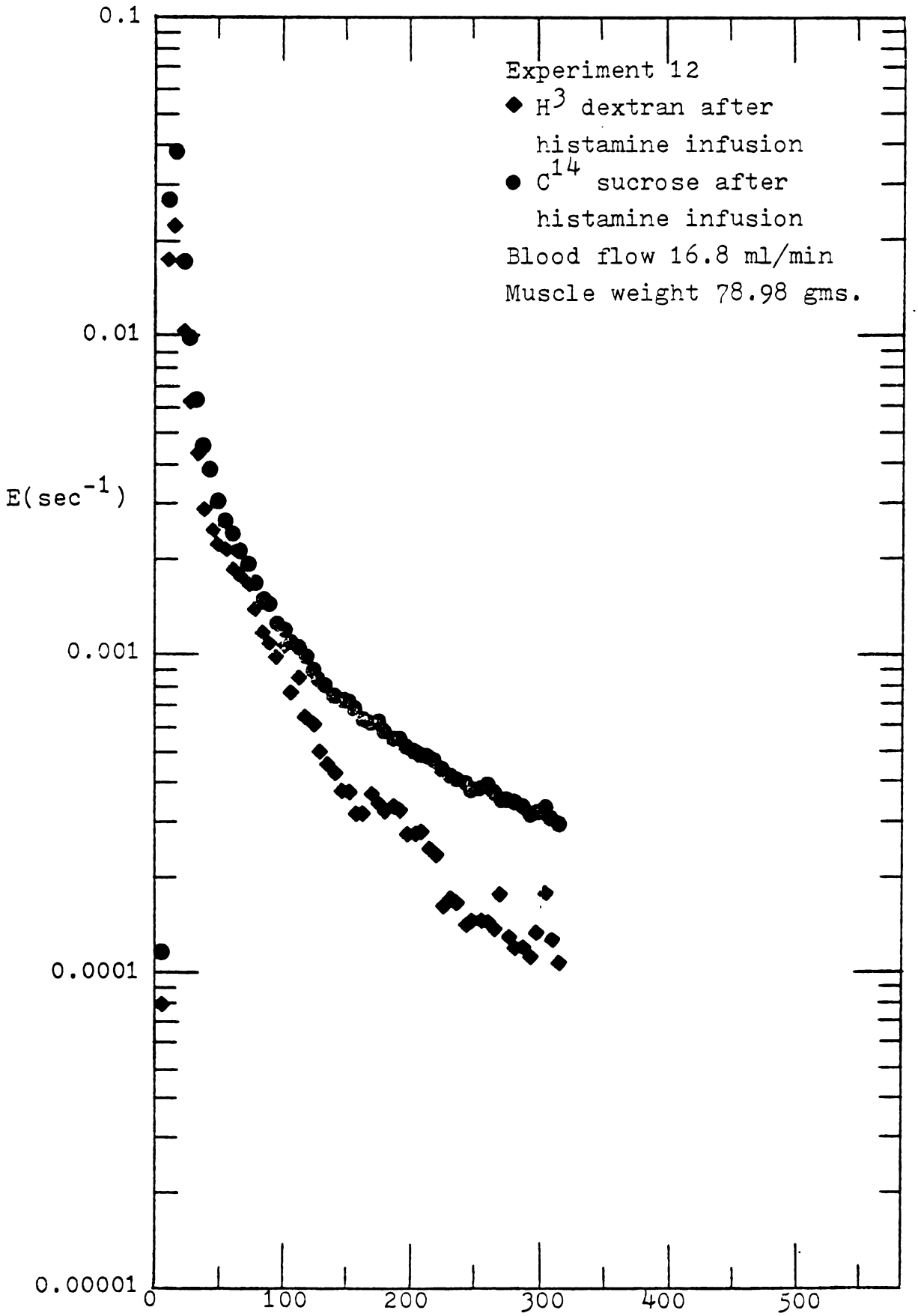



Figure 12c. Expt. 12

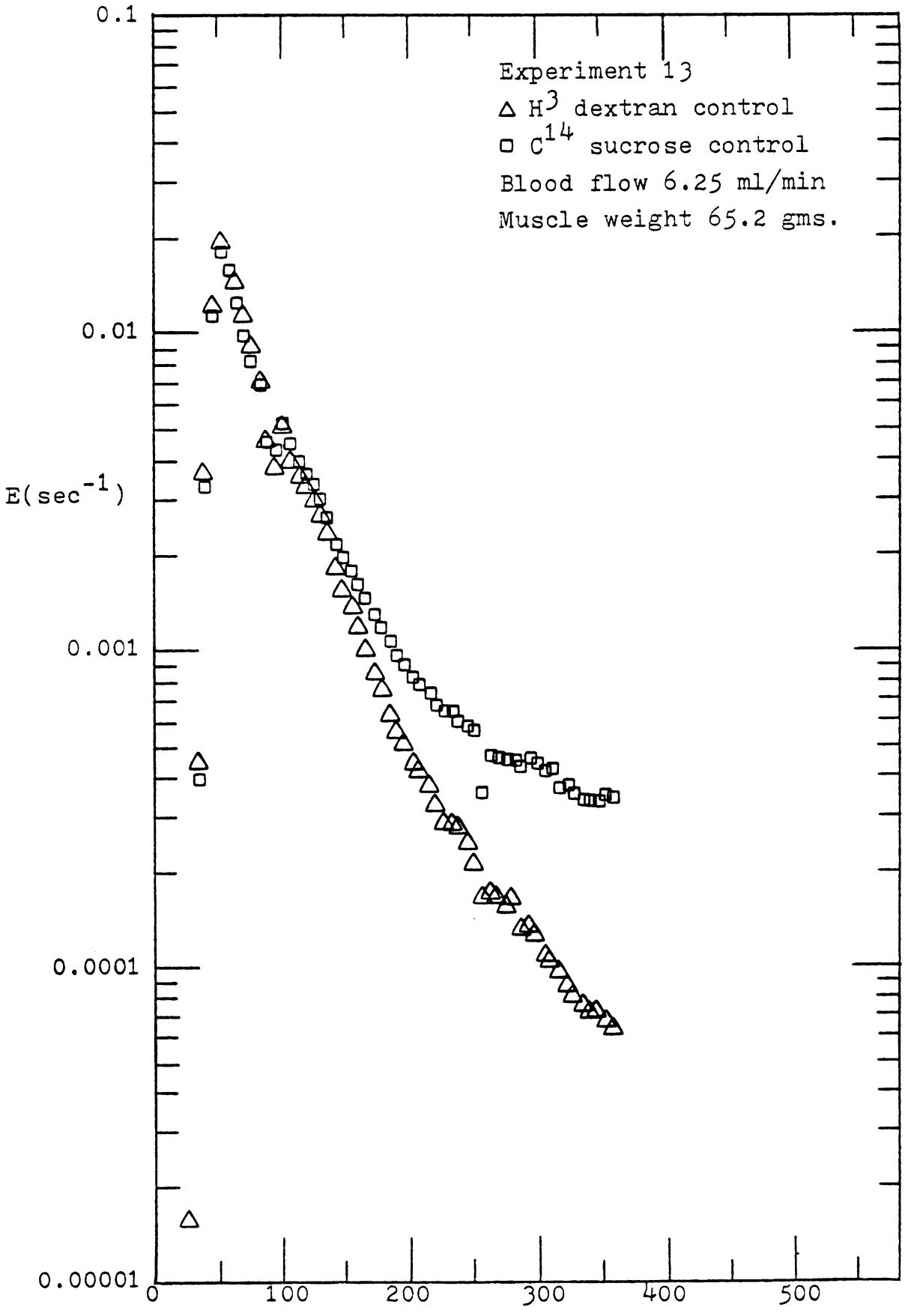



Figure 13a. Expt. 13

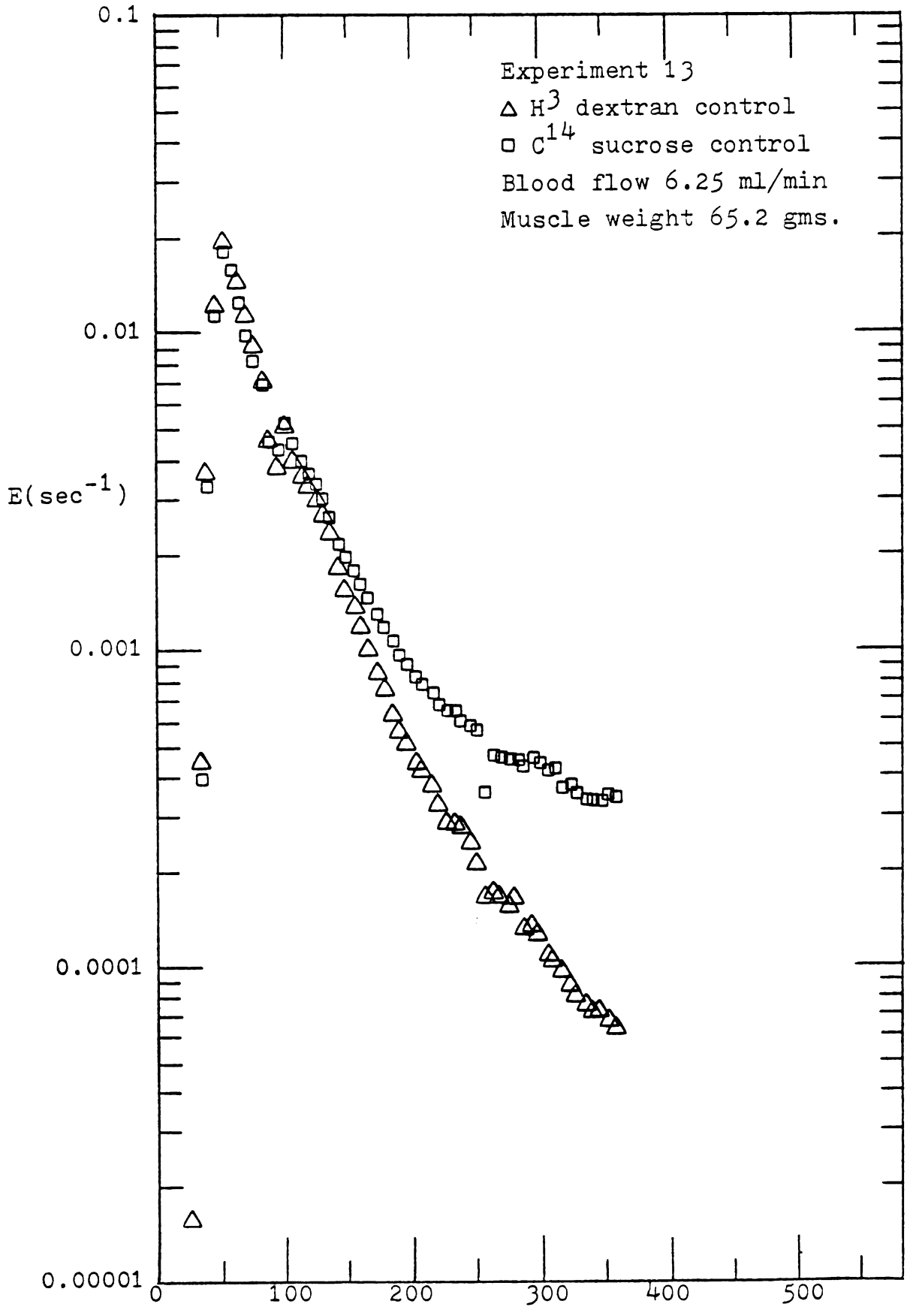



Figure 13a. Expt. 13

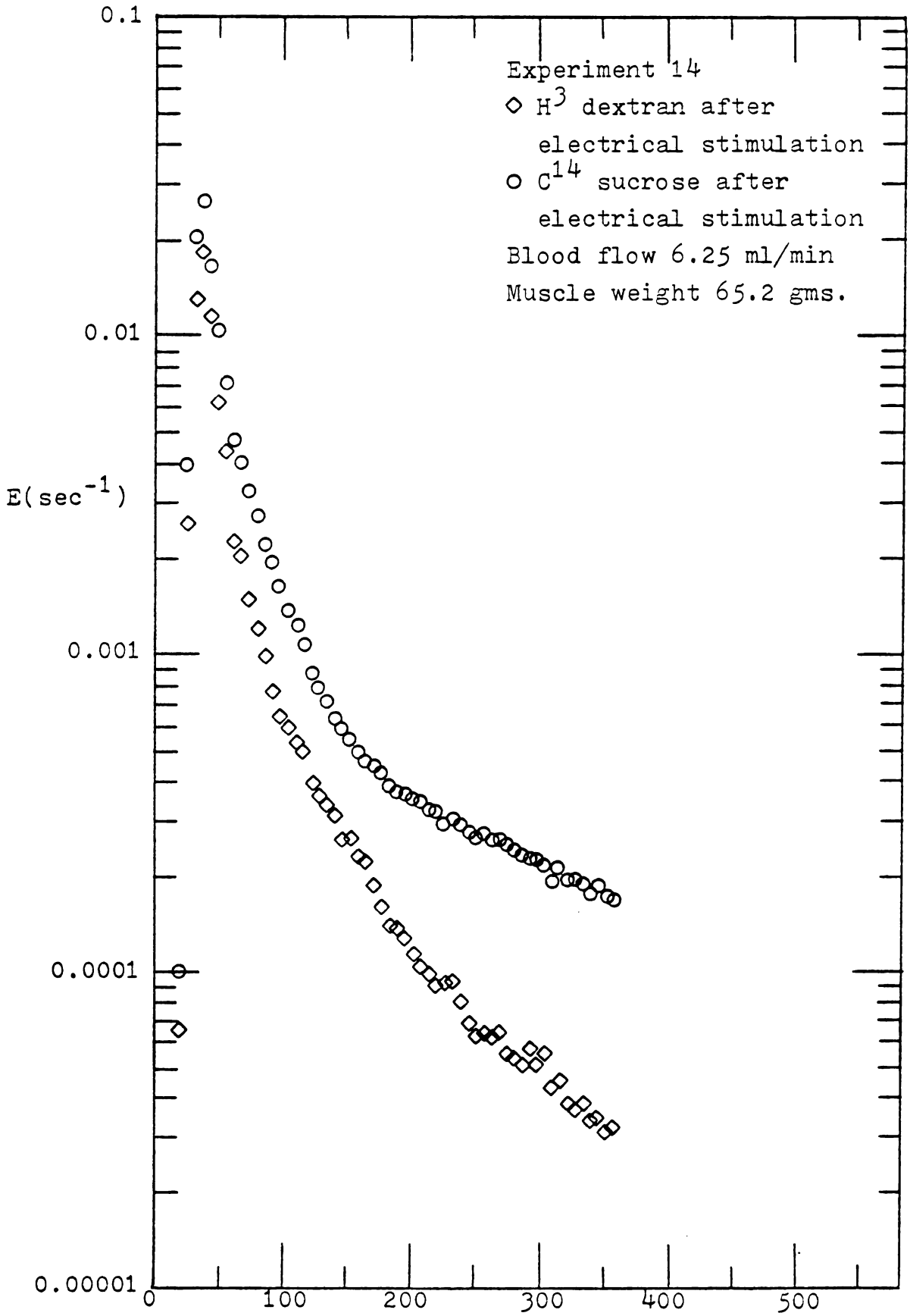



Figure 13b. Expt. 14

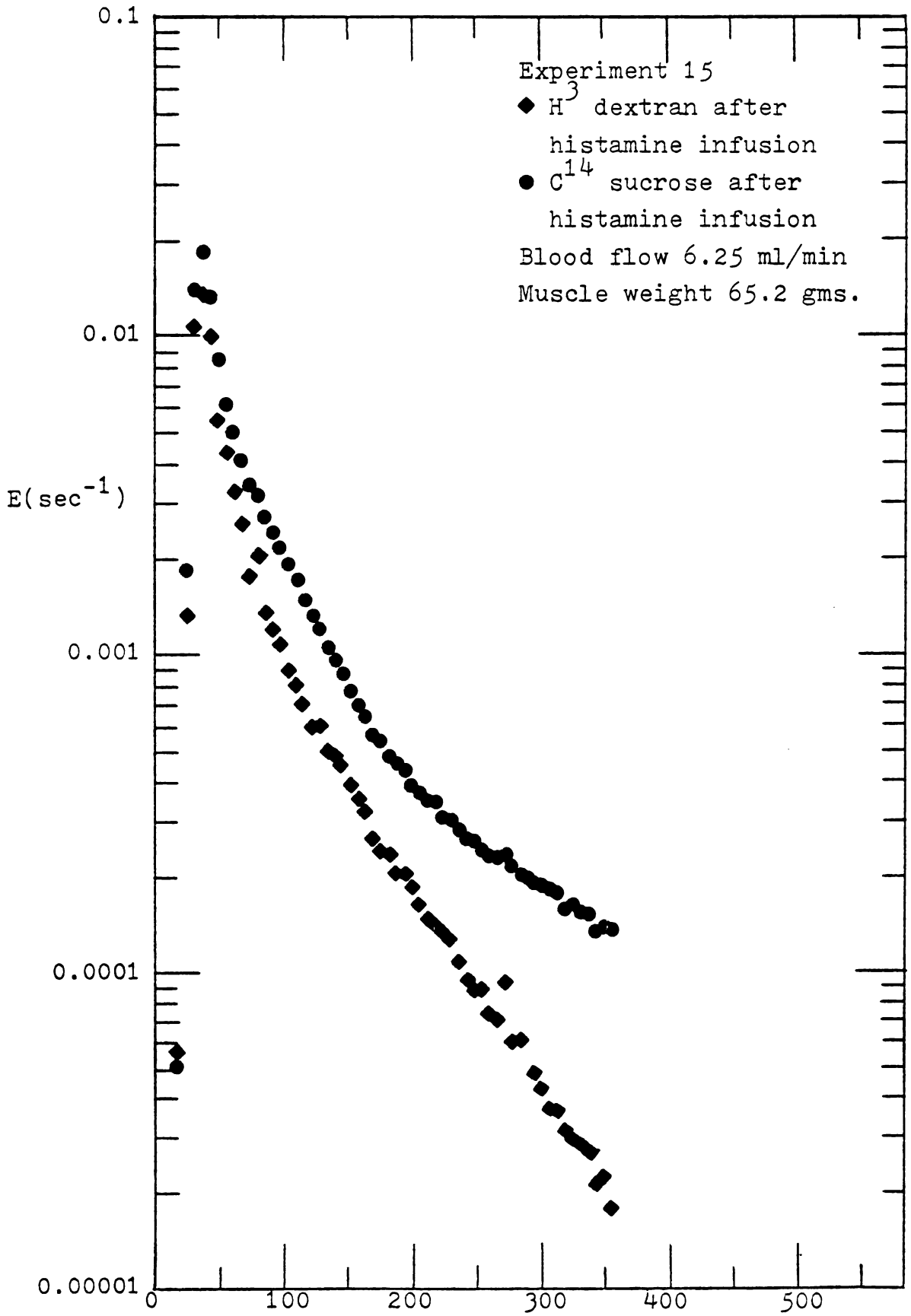



Figure 13c. Expt. 15

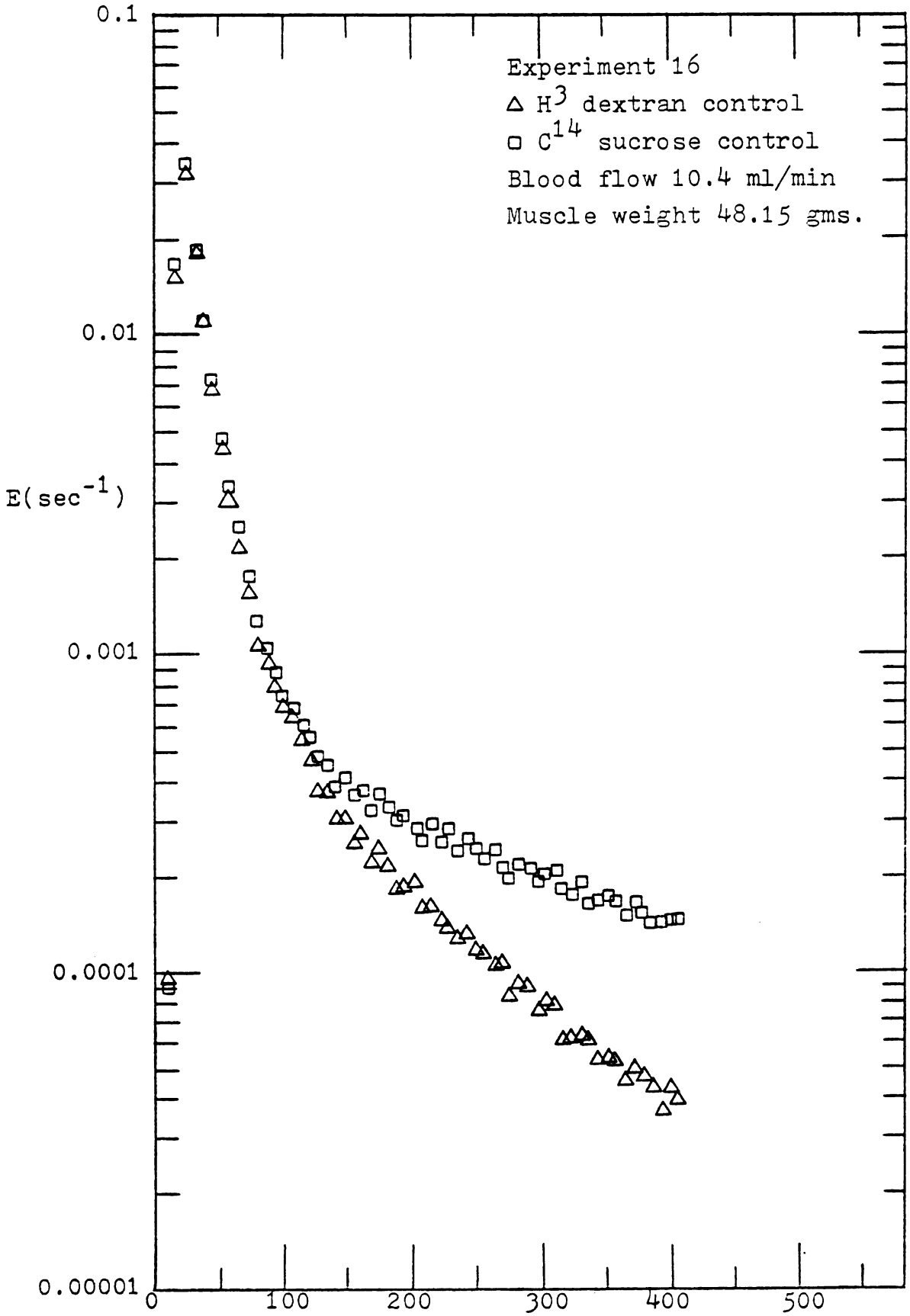



Figure 14a. Expt. 16

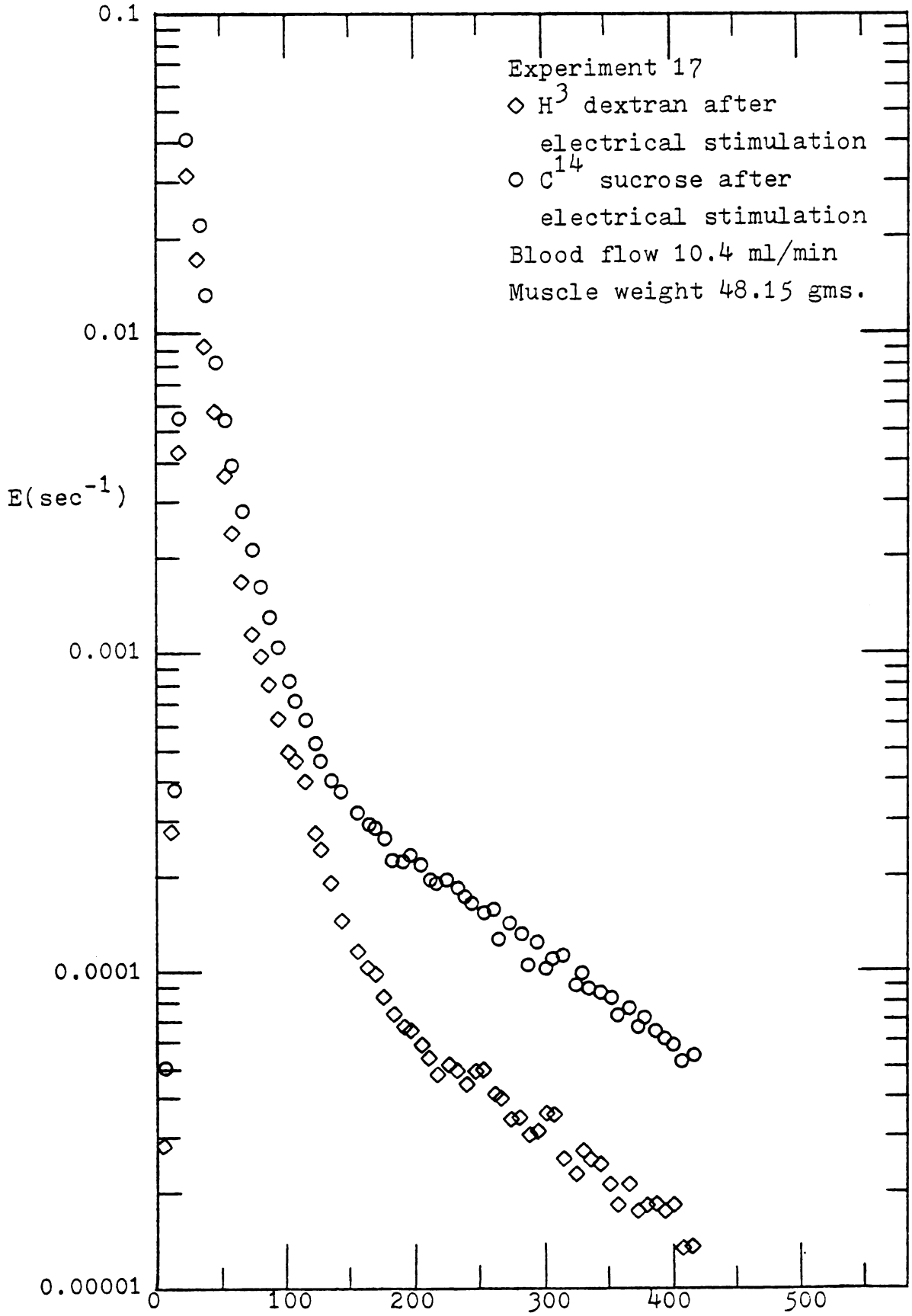



Figure 14b. Expt.17

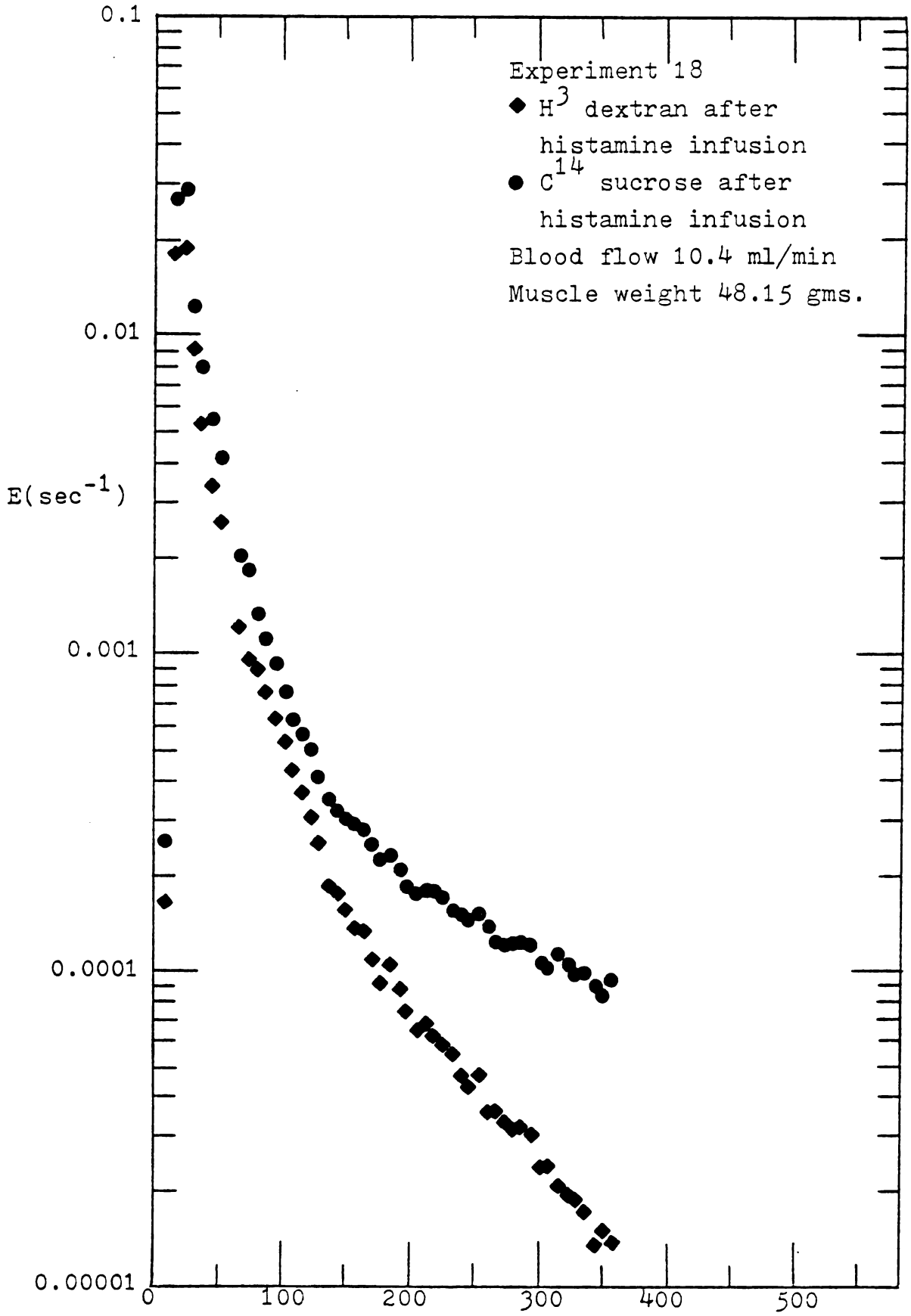



Figure 14c. Expt. 18

## SUMMARY AND CONCLUSIONS

Figures 4 through 14c show the E vs. time data obtained from the experiments. The obvious sign of parallel paths which might be seen on such a graph would be a distinct second peak in the data. In most of the graphs, no secondary peak could be seen, only a random fluctuation of one or two points at a time.

In Figure 5, however, there seems to be a distinct, well defined secondary peak. Whether or not this is due to a parallel path flow pattern is uncertain as this is the only graph which shows such a definite peak. Figure 12c shows what may be a second peak near the end of the experiment, but it is not as well defined as that in Figure 5.

It is suggested that many more experiments be performed with these same techniques and studied to see if any more of them yield a second peak in the data.

Table 1 shows the recoveries of the tracers based on a trapezoidal numerical integration of the E vs. time curves. Very little pattern can be seen in these values. In the dextran recoveries, the control values are highest followed by the values after electrical stimulation. The lowest values were those obtained after histamine infusion. The only consistent pattern in the sucrose recoveries is that the values obtained after the histamine infusion were the lowest. No consistent patterns are seen when comparing the sucrose and dextran recoveries. The fact that, in both the dextran and sucrose, the values obtained after the histamine infusion

are the lowest may be due to the effect of histamine on the capillary membrane. Histamine causes the membrane to become much more permeable (12); thus allowing a high rate of diffusion into the extracellular space where it may not have returned to the capillary system readily to have been detected.

One possible reason why there is no clearcut pattern to the tracer recoveries is seen when one looks at the factors determining the accuracy of those numbers. The accuracy of the individual E values depends on three items: the accuracy of the scintillation counting, the reproducibility of the volumes sampled, and a small loop volume i.e. small dt (see page 15). These criteria were fairly well met and the resulting data graphs were for the most part fairly smooth, much more so than those obtained by previous methods (4).

However, the accuracy of the tracer recoveries was affected by not only the accuracy of the individual E values, but also by having a sufficient number of points to insure that the entire graph was well defined. For example, if a sample was taken just before the concentration peaked and the next point was taken after that peak, there would be an area which was not counted in the recovery, one which may result in a significant error since it is in the region having the highest E value. If, in another experiment, a sample was taken right at the peak, a good representation of that area could be calculated and added to the tracer recovery. This value could not really be compared to the recovery calculated when

the peak E was missed. In addition, if any sharp secondary peaks exist and are missed by the sampling techniques, then additional error will follow.

If the scintillation counting sensitivity or the tracer activity could be increased, the sample loop volume could be decreased. This would have two results. It would lower the value dt and give greater accuracy to the individual E values and it would allow more samples to be taken since the time between samples could be decreased. These two factors combined would result in the calculation of more accurate recoveries.

Further experiments using these same techniques are suggested with possible refinements in the scintillation counting using perhaps a more sensitive machine or a higher tracer activity. In addition, experiments performed on vascular beds known to have shunting pathways, such as the skin of the canine paw (13), are highly recommended to see if any obvious signs of channeling are seen in the resulting E vs. time curves.

# LIST OF REFERENCES

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