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TEMPERATURE DEPENDENCE OF VENOM  
PHOSPHOLIPASE A AND RELATED HAEMOLYSIS

By

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JUNE 1969

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## ABSTRACT

Phospholipase A activity of the venom of Crotalus adamanteus was found to increase by a factor of two for every 10° C. increase in temperature. A percentage of haemolysis of red cells by lysolecithin produced by phospholipase A occurred at two times lower lysolecithin concentration for every 10° C. decrease in temperature. Under all conditions, percent lysis increased with decreasing temperature, although initially the temperature dependence is small. At any time, decreasing the temperature in a complex reaction mixture would be expected to produce an instantaneous increase in the percentage of cells lysed.

APPROVED:



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Commanding

## TEMPERATURE DEPENDENCE OF VENOM PHOSPHOLIPASE A AND RELATED HAEMOLYSIS

### INTRODUCTION

The use of cryotherapy in the treatment of snakebites was recently revived by Stahnke, Allen, Horan and Tenery (1957). Later, Ya and Perry (1960) performed experiments with dogs which indicated that the technique was not useful. Furthermore, McCollough, Grimes and Gennaro (1961) cited statistics which seemed to indicate that use of cryotherapy in the treatment of snakebite resulted in an increase in post-treatment disability. Nevertheless, Lockhart (1965) more recently suggests that the best course of treatment involves long-term cryotherapy. In fact, he argues that long term cryotherapy decreases permanent disability. We cannot help but conclude that cryotherapy is a controversial method of treatment.

The "scientific" basis for cryotherapy is that most enzyme activities are decreased by a factor of two for every  $10^{\circ}$  C. decrease in temperature. Hence, it is argued, less tissue damage should be expected at decreased temperatures. The problem must be more complicated than this, as questions of enzyme deterioration, constriction of blood vessels, etc. need to be considered. We do not wish to debate the use of cryotherapy. We wish to attack the premise that tissue damage should be decreased at temperatures lower than  $37^{\circ}$  C. We base this argument on the fact that the most significant damage is the result of the action of phospholipase A and the subsequent action of the product of this enzyme, lysolecithin, on membrane structures. We shall show that under certain conditions, initiation of cryotherapy might be expected to be damaging if similar in vivo behavior could be expected to our results in vitro.

### PHOSPHOLIPASE A ACTIVITY AS A FUNCTION OF TEMPERATURE

Venom phospholipase A was reviewed by Condrea and deVries (1965). All measurements mentioned failed to consider temperature dependence, but were conducted at physiological temperature. We studied the effect of temperature on the activity of Eastern Diamondback Rattlesnake (Crotalus adamanteus) venom essentially using the method of Kocholaty (1966). The experimental procedure follows:

1. Fresh venom was removed from one rattlesnake and used within an hour. We had previously observed significant decrease in potency on standing in concentrated form even at 0° C. Simultaneous measurements were made at 13° C. with venom diluted 1/1250 and 1/2500, at 25° C. with venom diluted 1/2500 and 1/5000, and at 37° C. with venom diluted 1/5000 and 1/10000. The dilutions stated are those in the reaction mixtures.

2. A blank was determined after 2 hours at each temperature. The values of all blanks were the same. An infinite time value was determined with minimally diluted venom after two hours at 37° C. The final titer values (explained below) were 0.2 and 1.6 ml for blank and infinite time runs respectively.

3. Each reaction mixture consisted of 2 parts 0.1 M. TrisOH plus HCl, pH 7.8, 0.0005 M. CaCl<sub>2</sub>, 1 part of 0.2 M. TrisOH plus HCl, pH 7.8, 0.001 M. CaCl<sub>2</sub>, 1 part egg yolk. 1.9 ml of this mixture plus 0.1 ml venom at 20 times the desired final concentration in 0.15 M. NaCl were separately taken to temperature in a constant temperature bath and mixed at time zero.

4. The reaction was stopped at time  $t$  by addition of 10 ml of a 40/10/1 (v/v/v) mixture of isopropanol/heptane/1.0 N H<sub>2</sub>SO<sub>4</sub>. The reactions containing the higher concentration of venom were stopped at 15, 30, 60, 90 and 120 minutes. The reactions containing the two-fold dilution of venom were stopped at 30, 60, 120, 180 and 240 minutes.

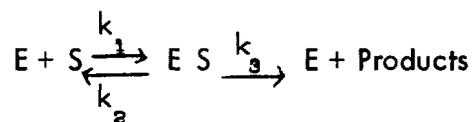
5. The following procedure was then followed with each sample: Add 4 ml water. Add 6 ml heptane and mix. Allow the mixture to settle.

6. Take 3 ml of the upper phase. Add 3 ml isopropanol. Add 1 ml 0.3% thymol blue in ethanol.

7. Titrate to the end point (by color matching) with 0.01 M. ethanolic sodium hydroxide and record the volume of titrant.

Our results at 37° C. are consistent with those of Kocholaty (1966) using a titrimeter. Although the color produced at the end point is not stable over a period of time, titration of aliquots of the same sample leads to similar results.

From steady state enzyme kinetics for



where  $k_i$  are the rate constants,  $S$  is substrate, in this case lecithin,  $E$  is enzyme, in this case phospholipase  $A$ , we may derive a form of the Michaelis-Menton equation

$$v = - \frac{d[S]}{dt} = \frac{k_3 [E_{\text{total}}] [S]}{\frac{k_2 + k_3}{k_1} + [S]}$$

where  $[E_{\text{total}}] = [E] + [ES]$

From this observation that the rate depends on  $S$ , we may conclude either that

$$\frac{k_2 + k_3}{k_1} \approx [S] \quad (\text{Michaelis-Menton kinetics})$$

or  $\frac{k_2 + k_3}{k_1} \gg [S] \quad (\text{First Order Kinetics})$

We tested the latter hypothesis and found that our data was sufficiently in agreement to allow this simplification. The rate constants thus obtained are in reciprocal seconds and enzyme concentration, eliminating the need to consider the concentration of substrate.

$$\text{For } v = - \frac{d[S]}{dt} = \frac{k_1 k_3 [E_{\text{total}}] [S]}{k_2 + k_3}$$

$$\text{Log}_{10} \left( \frac{ml_{\infty} - ml_0}{ml_{\infty} - ml_t} \right) = \frac{k_1 k_3 [E_{\text{total}}]}{2.303 (k_2 + k_3)} t$$

where  $ml_0$ ,  $ml_t$  and  $ml_{\infty}$  are the milliliters of titrant necessary for reactions at time zero (blank),  $t$  and time infinity.

$$\text{Define } [E_{\text{total}}] = D [E_{\text{undiluted}}]$$

where  $D = \text{dilution factor}$

$$\text{Log}_{10} \left( \frac{ml_{\infty} - ml_0}{ml_{\infty} - ml_t} \right) = K D t$$

$$\text{where } K = \frac{k_1 k_3 [E_{\text{undiluted}}]}{2.303 (k_2 + k_3)}$$

The complex rate constant,  $K$ , may be determined from a semilogarithmic plot of

$$\frac{ml_{\infty} - ml_0}{ml_{\infty} - ml_t} \quad \text{versus} \quad D t$$

and half-times for digestion of lecithin,  $t_{\frac{1}{2}}$ , for a given dilution factor,  $D$ , is given by

$$D t_{\frac{1}{2}} = \log 2/K$$

Results for the experiment described above appear in Figure 1.

FIRST ORDER RATE PLOTS FOR DIGESTION OF LECITHIN BY VENOM PHOSPHOLIPASE A

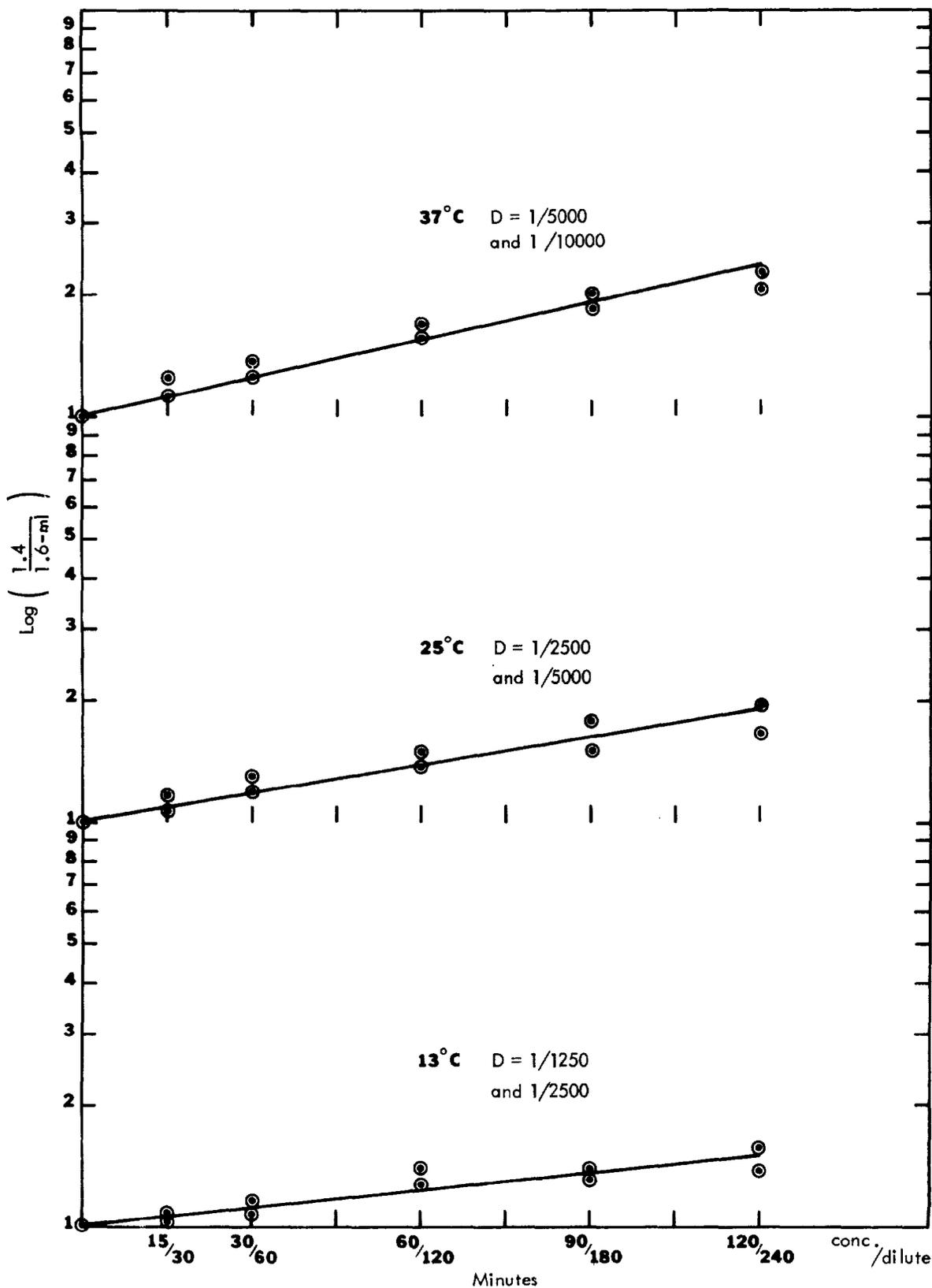


FIGURE 1

We have further observed that in the temperature range considered, a simple temperature coefficient may be calculated.

$$\log \frac{K_2}{K_1} = - \frac{\Delta H}{2.303 R} \left( \frac{1}{T_2} - \frac{1}{T_1} \right)$$

where  $T_i$  are temperatures in degrees Kelvin and R is the gas constant.

Figure 2 shows the results of such a calculation.

$$\Delta H = 15.3 \text{ K Cal/mole}$$

The sign and magnitude of the enthalpy are similar to those commonly observed in enzymatic systems. The enzyme activity increases about a factor of 2 for every 10° C. increase in temperature.

#### HAEMOLYSIS BY LYSOLECITHIN AS A FUNCTION OF TEMPERATURE

Robinson (1961) most recently reviewed the subject of lysolecithin. Collier (1952) performed studies of the temperature dependence of haemolysis similar to the experiments reported in this section of this report. Our results reach the same qualitative conclusion that decreasing temperature increased lysis at a given lysolecithin concentration. Quantitatively, we find a larger temperature coefficient than did Collier (1952). We also failed to observe the same kind of kinetic effects as he reported. Lysis at low temperatures could be seen with the unaided eye to occur almost instantly with mixing of cells with lysolecithin. Waiting for 30 minutes at 13° C. and 25° C. had less than a 30% effect on the required concentration of lysolecithin to produce and observed percentage of lysis. More effect was observed at 37° C. presumably because of continued production of lysolecithin. Venom alone would not lyse red cells under these conditions. The procedure used in our experiments is given below:

1. Fully digested egg yolk was prepared according to the procedure used for preparation of infinite time samples in the previous section.
2. Red cells were washed two times in 0.15 M. NaCl, 0.02 M. TrisOH plus HCl, pH 7.8.
3. A 1/1 mixture of some dilution of lysolecithin solution into 0.15 M. NaCl, 0.02 M. TrisOH plus HCl, pH 7.8 and of red cell solution was

DETERMINATION OF  $\Delta H$  FOR DIGESTION OF LECITHIN BY VENOM PHOSPHOLIPASE A

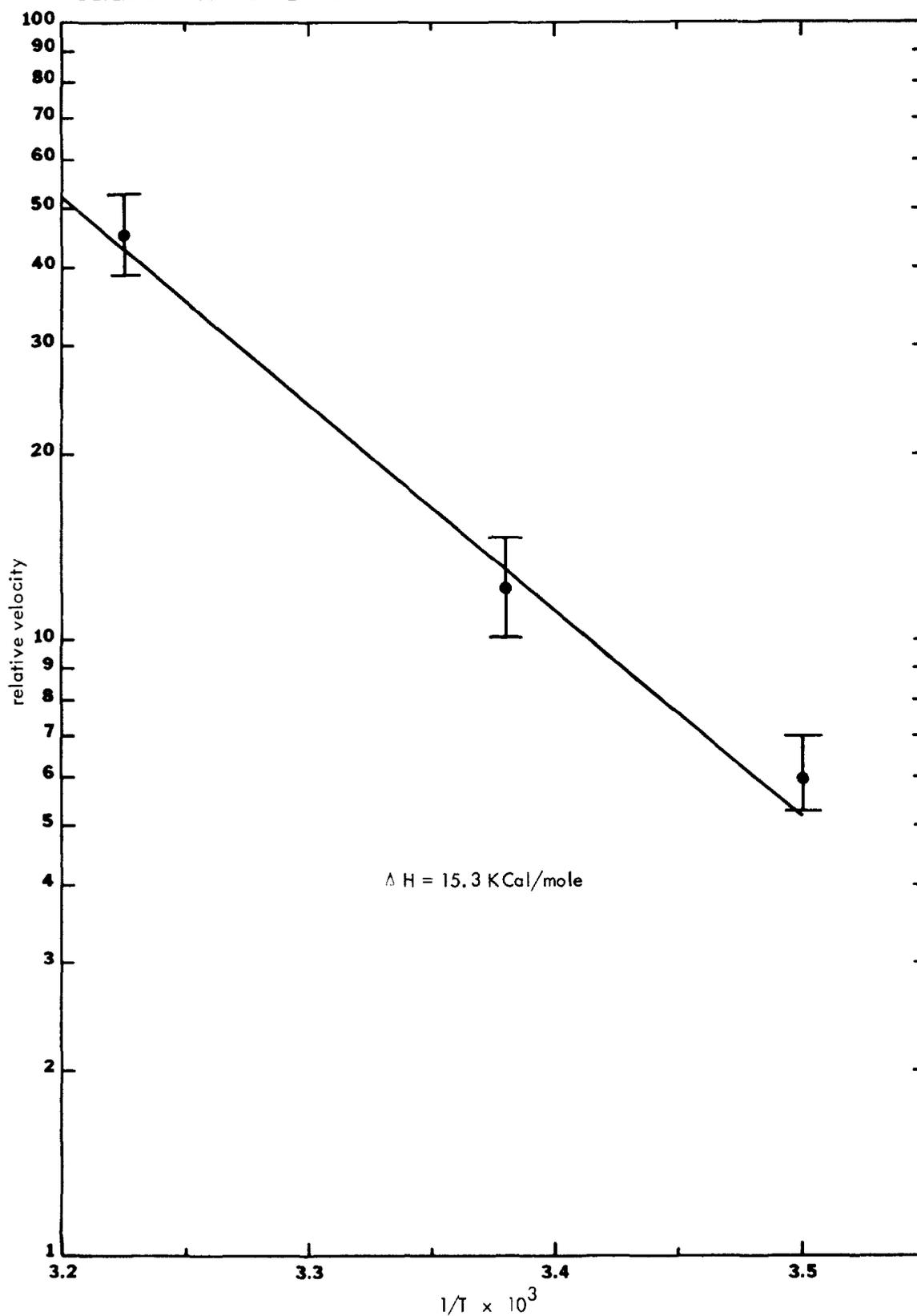


FIGURE 2

made from the individual solutions preincubated at the desired temperatures. The mixture was gently rocked for a few seconds.

4. The remaining unlysed red cells were pelleted in a clinical centrifuge by spinning for 4-5 minutes.

5. The supernatant was observed at 420 milimicrons after necessary dilutions were accomplished.

Figure 3 shows the percent lysis as a function of percent lysolecithin at three temperatures, 13°, 25° and 37° C. The results clearly show that lysolecithin alone will lyse red cells much more easily at decreased temperatures. Lysis occurs at approximately 2 times lower lysolecithin concentration for every 10° C. decrease in temperature. Thus, the overall effect of times at varying temperatures in a system of venom, serum lecithin substrate and cells is difficult to predict.

## CONCLUSIONS

1. The initial overall haemolysis rate as a result of the two sequential reactions is the same at every temperature studied.

2. As lysolecithin concentration becomes larger and lecithin concentration decreases, an overall negative temperature coefficient appears. Under all conditions, the overall haemolysis at one temperature tends to increase relative to the overall haemolysis at a higher temperature as the reaction proceeds.

3. At any time, decreasing the temperature leads to an instantaneous and sustained increase in haemolysis relative to results at constant temperature.

4. At any time, increasing the temperature leads to a period of inactivity in terms of haemolysis followed by a delayed normal dependence of lysis on temperature.

5. The argument for decreased tissue damage due to treatment of snakebite by cryotherapy which relies on a decrease in enzymatic effects at lower temperatures cannot be considered to be correct.

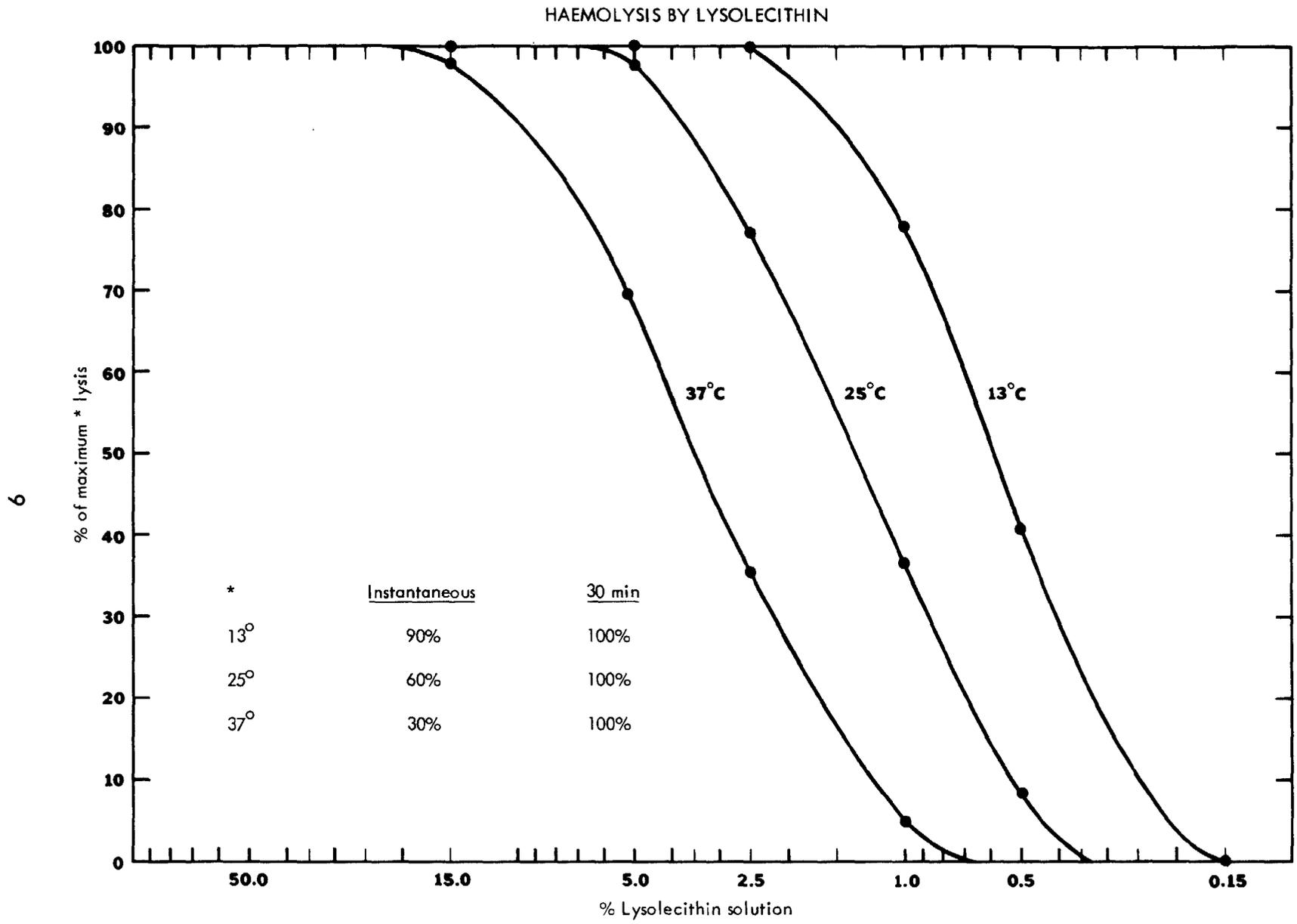


FIGURE 3

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