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Hemolysis in the Erythrocytes of Frogs with Red Leg Disease

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HEMOLYSIS IN THE ERYTHROCYTES OF FROGS WITH RED LEG DISEASE

by

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Abstract

Hypotonic Hemolysis was induced in the erythrocytes of frogs with red leg disease. The disease causes a decrease in red cell count and hemoglobin levels.

Per cent hemolysis measured in these frogs corresponds very well with those of normal (non-diseased) frogs (Jacobs, et al., 1951). This suggests that fragility of the cell membrane is not increased in this disease. The erythrocyte in response to hemolysis gave results which were indicative of a non-linear relationship.

Introduction

Per cent hemolysis in the mammalian erythrocyte has been measured for many years by numerous investigators studying cell permeability (Ponder, 1948). Among the significant accomplishments are the results of studies on the effects of ultraviolet radiation on the mammalian erythrocyte permeability (Leu, Wilbrandt, and Liechti, 1942; Green, 1956; Cook, 1956).

Hemolysis rate for the amphibian erythrocyte has received less attention (Ponder, 1948) even though large variations in red cell permeability have been demonstrated among lower vertebrate species (Jacobs, Glassman, and Parpart, 1951). The presence of a nucleus, the active oxidative metabolism (Hunter and Hunter, 1957), and its contribution to the maintenance of a cation transport system (Maizels, 1954) sufficiently distinguish the nature of differences between lower vertebrate erythrocytes and mammalian red blood corpuscles to warrant further study of these lower vertebrate cells. Some work has been done on frog erythrocytes and this involved the use of ultraviolet radiation to induce hemolysis (Maroney, 1960).

In the present investigation the erythrocytes of frogs with red leg disease were treated with varying concentrations of hypotonic saline solutions and the observed percentages of hemolysis were recorded. The disease, red leg, is a generalized inflammation due to irritation by chemicals, rough grounds, etc. (Kaplan, 1952). These frogs were chosen because this disease occurs

frequently and tends to lower the red cell count in both males and females (Kaplan, 1952). The hemolysis rate has not been measured in these infected frogs. Frogs with this disease contain a large number of vacuolated red cells and exhibit a decrease in hemoglobin levels as well as hematocrit (Kaplan 1952).

Methods and Materials

Instruments for dissection, centrifuge tubes, and cuvets were sterilized for use with each new specimen so that hemolysis of cells by an external source could be eliminated. The frogs were then sexed, weighed, and their length determined before beginning the following procedure.

Leopard frogs (Rana pipiens) were anesthetized with 2 ml. of urethan (5% solution) injected into the dorsal lymph sac. Complete anethesia was affected in 5 to 10 minutes.

After careful incision into the thoracic cavity, blood was drawn from the ventricle of the heart with a heparinized syringe and a 20 or 22 gauge needle. About 0.1 ml. of blood was put into tubes containing 5 ml. of hypotonic saline solutions of constant temperature (24° C) and increasing concentrations, namely, .008 M., .017 M., .034 M., .051 M., .068 M., .085 M., and .102 M respectively. These volumes and concentrations gave the most reliable readings of per cent hemolysis in these diseased frogs. Per cent hemolysis was recorded at 5, 15, and 60 minute intervals. The tubes with the blood and varying concentrations of hypotonic saline solution were centrifuged at 1000 g for 5 minutes. The supernatants were then poured into cuvets for analysis of per cent transmittance[1]

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[1]Ratio of radiant power transmitted by a sample (P) to radiant power incident on a sample (P_o), both being measured at the same spectral position (wavelength) and with the same spectral band width: $T=P/P_o$ and $\% T=100T$.

in a Spectronic "20" spectrophotometer at a wavelength of 540 mμ (determined best for Hb absorption). Per cent transmittance was changed to per cent hemolysis by using a standardized curve for hemoglobin levels in erythrocytes (Henry, 1967).

The standard curve is a semilog plot of per cent transmittance vs. gm.% of hemoglobin in a sample. This standard curve is set up by using increasing dilutions of Hycel Cyanmethemoglobin Standard. For the 5.0 ml. volume employed in the unknown samples the undiluted standard corresponds to 20 gm.% hemoglobin. Dilutions with Hycel Cyanmethemoglobin Reagent[1] were made to correspond to 15 gm.%, 10 gm.%, 5 gm.%, and zero to obtain the curve.

TABLE I

The different volumes of standard and reagent give a corresponding change in the gm.% of hemoglobin.

Gm.% Hb	20	15	10	5	blank
Vol. of Standard (ml.)	6.0	4.5	3.0	1.5	none
Vol. of Reagent (ml.)	none	1.5	3.0	4.5	6.0

In utilizing the standard curve, the gm.% of hemoglobin is known and a point of complete (100%) hemolysis is obtained from the

[1]Consists of 1.0 gm. $NaHCO_3$, 50 mg. KCN, and 200 mg. $K_3Fe(CN)_6$ per liter of water. Usually good for one month when stored in a dark bottle out of the light.

most hypotonic solution (.008 M. NaCl). Complete hemolysis lies on a point of the curve obtained from Table 1. This is then equated to the total Gm% of hemoglobin in the system being utilized.

Following the procedures for obtaining per cent hemolysis, a hematocrit was obtained using heparinized capillary tubes and an International Micro-hematocrit centrifuge and reader (compares volume of cells to total volume of blood). The volume per cent of cells was used as an indication of the volume of cells in a blood sample of the frogs which had red leg disease.

Results

The mean per cent[1] hemolysis, the corresponding concentrations of saline solution, and the time intervals are presented in Table 2.

Table 2

Influence of concentration of saline solution and time on hemolysis[1,2] in frogs with red leg disease.

(Molar) NaCl Concentration	Recording Intervals for per cent Hemolysis[2] 5 min.	15 min.	60 min.
.008	90.00	97.00	100.00
.017	70.00	79.00	86.00
.034	54.00	61.00	67.00
.051	36.00	42.00	48.00
.068	12.00	16.00	21.00
.085	1.80	1.80	3.40
.102	0	.04	.06

If concentration and time are increased the observed per cent hemolysis increases. Likewise, when the time is held constant, and the concentrations of saline solutions are increased, the per cent hemolysis will decrease up to an isosmotic condition.

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[1]All values are based on 80 observations

[2]Values of per cent hemolysis are converted from per cent transmittance measured experimentally.

The curve (Fig. 1, appendix) fitted free hand to the observed points in Table 2, indicates a second degree relationship between per cent induced hemolysis and increasing concentrations of hypotonic saline solutions. This deviation from linearity is attributed to random factors. The predicted values ($\hat{H}$) for per cent hemolysis are obtained by assuming a straight line or linear regression of hemolysis on concentration. The equation for such a regression is given as:

$$\hat{H} = \overline{H} + b_{HC} (C-\overline{C}), \qquad (1)$$

where $\hat{H}$ is the hypothetical or predicted value for per cent hemolysis for different concentrations, $\overline{H}$ is the mean per cent hemolysis, b_{HC} is the regression of hemolysis on the changes in concentration, C and $\overline{C}$ are respectively the selected concentration and mean concentration of the saline solutions. The term $b_{HC} = \dfrac{(\Sigma C)(\Sigma H)}{N}$

$$\Sigma C - \frac{(\Sigma C)^2}{N}$$

and represents the average change in hemolysis per unit change in concentration (Steel and Torie, 1960). Values for the predicted per cent hemolysis correspond well at lower concentrations in comparison to those of solutions with greater concentrations (Table 3)[1] (Fig. 2, appendix).

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[1]Averaged over three time periods, namely 5, 10, and 15 minutes.

TABLE 3

Observed and predicted per cent hemolysis values averaged over three periods after dilution with saline solutions of varying concentrations.

Concentrations of saline Percentage	Per cent Hemolysis observed (H)	Predicted ($\hat{H}$)
0.05	96.00	88.14
0.1	78.00	79.28
0.2	61.00	61.00
0.3	42.00	43.49
0.4	16.00	26.23
0.5	2.00	8.54
0.6	.05	-9.15

In Table 3, H values are averages of per cent hemolysis of 80 samples, at a given concentration, for three time periods. The $\hat{H}$ values are obtained from equation 1 after obtaining the constants in the equation, e.g. ΣH is 295.05 (Table 4, appendix). The values estimated are from the linear fit and the nature of the plot is closer to the observed value at lower concentrations of saline (First degree relationship) than at higher saline concentrations. Therefore, equation 1, which is linear, fails to describe the situation accurately at certain concentrations, e.g. 0.5% and 0.6%. It is likely that a second degree equation would more accurately describe the response. When more data become available a second degree fit will be attempted.

Normal frogs have a hematocrit of 38% to 42% (Kaplan, 1952). Hematocrits obtained from the frogs with red leg vary between 10% and 19.5% depending on the severity of the disease. The hematocrit was an indication that blood cell volume was reduced by 37% to 45% from that of normal frogs.

Discussion

Results obtained in hypotonic hemolysis of mammalian erythrocytes by Hjelm, Östling, and Personn (1965) indicated that (1) enzymes and co-factors are at least partially free to move out of the cell and (2) there seems to be a tendency for molecular species with lower molecular weights or smaller molecular dimensions to escape at a higher rate from the cell. Hemolysis is, however, a complex process and the interpretation of "escape data" in terms of molecular properties of the escapants presents considerable difficulty.

The magnitude of loss of a substance will depend on the extent to which its movement is restricted spatially or temporally. It may be fixed to some structural part of the cell which will prevent some or all from escaping. It may also be complexed to some other component with the result that the complex escapes at a different rate. Independent of any restriction imposed by a barrier such as a membrane, differences in diffusion rates will mean that the smaller the molecule, the faster it will escape from the cell. As the hemolytic process is of a transient nature, the limited time available may effect differences in relative final concentrations in the cell. Furthermore this effect will be exaggerated since the transient increase in porosity also exerts a molecular sieve effect, e.g. as with a heteroporous membrane (Hjelm, et.al., 1965).

Increasing concentrations of saline solutions gave an almost linear decrease in per cent hemolysis. This observed deviation from

linearity is probably due to variable cell age in the population. The older cells being more fragile, tend to hemolyze more readily than younger erythrocytes. Concentrations of saline which proximate the isosmotic point have little effect on most of the cell population except for the older erythrocytes. Therefore, these older erythrocytes that hemolyze cause a constant amount of hemolysis and the curve then begins to level off. (Fig. 1, Appendix)

The predicted per cent hemolysis is obtained through the use of the linear regression equation (equation 1). This adjustment of per cent hemolysis allows the prediction of values for hemolysis ($\hat{H}$) before experimental values are obtained. These values obtained by using a linear regression (equation 1). could be valuable to a person wishing to induce hypotonic hemolysis in a system.

Frogs with red leg disease gave results (percent hemolysis) that were comparable to normal frogs (Jacobs, et.al, 1951). The decrease in hemoglobin levels of the cells of the diseased frogs necessitated the use of a relatively large volume of blood (0.1 ml.) for each concentration of saline. This, however, did not effect the results because approximately the same number of cells were used for each sample.

Interpretation of the data indicate that the fragility of the erythrocyte cell membrane is not increased by red leg disease. Some questions still remain to be answered. First, the effect of increased white cell count (red leg disease) on the remaining erythrocyte population must cause a stress condition because there

are fewer erythrocytes to carry oxygen. Secondly, the presence of vacuoles in the erythrocytes of these diseased frogs (Kaplan, 1952) suggests a decrease in the amount of oxygen carried by the diminished volume of hemoglobin in their erythrocytes. And finally, the affect of a decrease in oxygen suggests the partial, if not total, loss of some oxidative processes in the erythrocytes of frogs with red leg disease.

Literature Cited

Carlson, Paul E. and Alvin R. Tarlov, 1962. Biochemistry of hemolysis. Ann Rev. Med., 13: 105-126.

Cook, J. S. 1956. Some characteristics of hemolysis by ultraviolet light. J. Cell. and Comp. Physiol., 47: 55-84.

Green, J. W. 1956. The separation of cation exchange and glycolysis in human red cells opposed to non-ionizing radiations. J. Cell. and Comp. Physiol., 47: 125-136.

Henry, Richard J., 1967. Clinical Chemistry: Principles and Techniques. Harper and Row, New York, pp 737-747.

Hjelm, Magnus, Persson. 1966. The loss of certain cellular components from human erythrocytes during hypotonic hemolysis in the presence of dextran. Acta Physiol. Scand. 67 (1): 43-49.

Hunter, A. S. and F. R. Hunter. 1957. A comparative study of erythrocyte metabolism. Ibid., 49: 479-502.

Jacobs, M.H., H.N. Glassman and A.K. Parpart. 1950. Hemolysis and zoological relationship. Comparative studies with four penetrating nonelectrolytes. J. Exp. Zool., 113: 277-300.

Kaplan, H.M. 1952. A study of frog blood in red leg disease. Trans. Illinois State Acad. Sci. 44: 209-215.

Leu, J.W., W. Wilbrandt and A. Liechti. 1942. Untersuchungen Uber Strahlenhamolyse. 3 Mitterlung: Ultraviolett-hamolyse. Strahentherapie, 71: 487-506.

Maizels, M. 1954. Active cation transport in erythrocytes. Soc. Exp. Biol. Symp., 8: 202-227.

Maroney, Samuel P., Jr. 1960. Ultraviolet-induced hemolysis of frog erythrocytes in the presence of various nonelectrolytes.

Ponder, E. 1948. Hemolysis and Related Phenomenon. Grune and Stratton, New York.

Steel, G. D. and J. H. Torie. 1960. Principles and Procedures of Statistics. McGraw-Hill, N. Y.

Appendix

TABLE 4

Calculated values from concentrations and observed hemolysis

Concentrations of saline (%)	Observed % Hemolysis (H)	Predicted % Hemolysis (H)
.05	96	88,14
0.1	78	79,28
0.2	61	61,60
0.3	42	43,49
0.4	16	26,23
0.5	2	8,54
0.6	,05	-9,15

Calculated:

$C = 2,15$ $\quad \Sigma H = 295,05$

$\bar{C} = .307$ $\quad \bar{H} = 42,15$

$\sigma^2 \quad .9125$

$$\Sigma CH - \frac{(\Sigma H)(\Sigma C)}{N} = \Sigma ch$$

Fig. 1

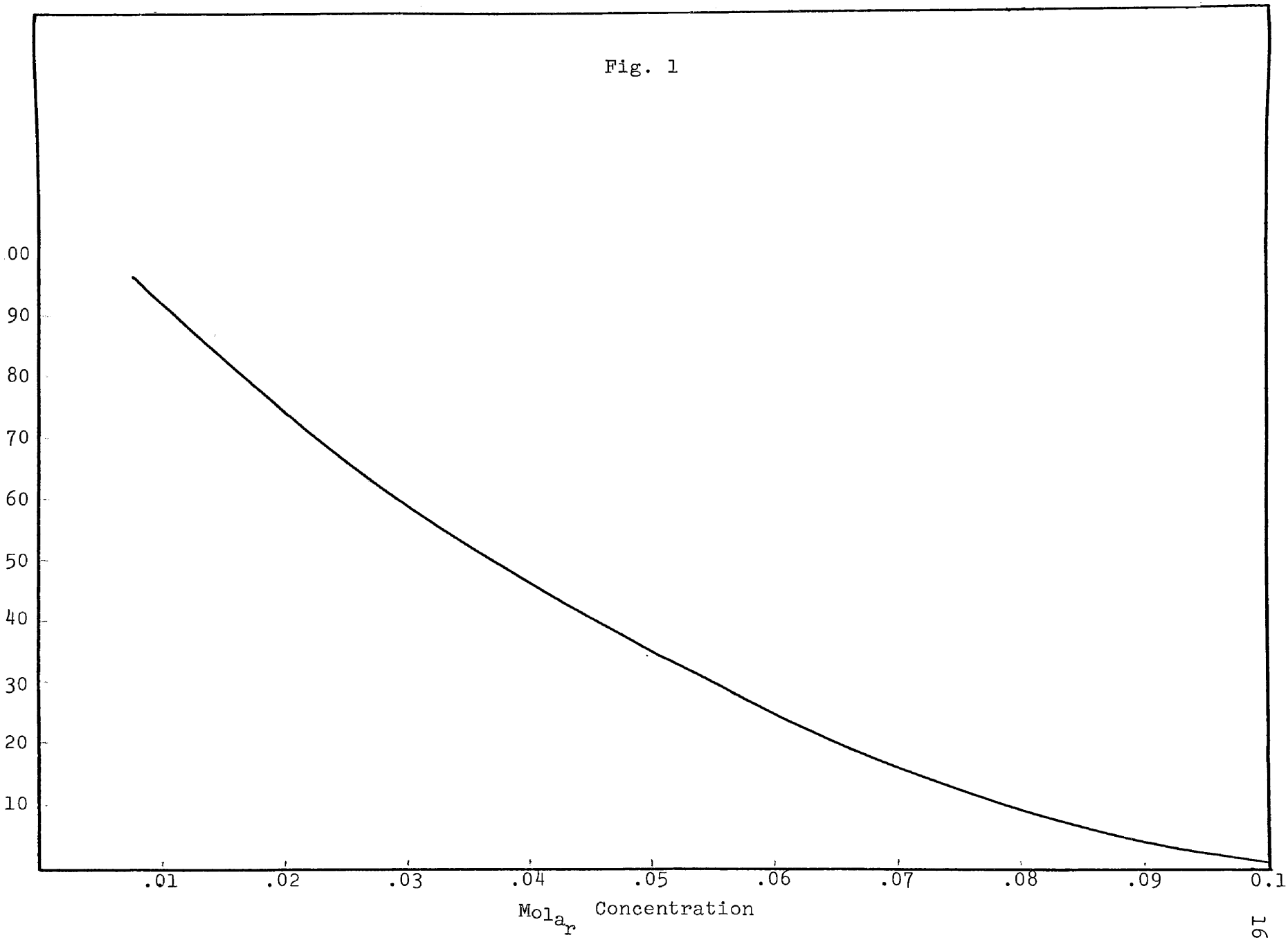
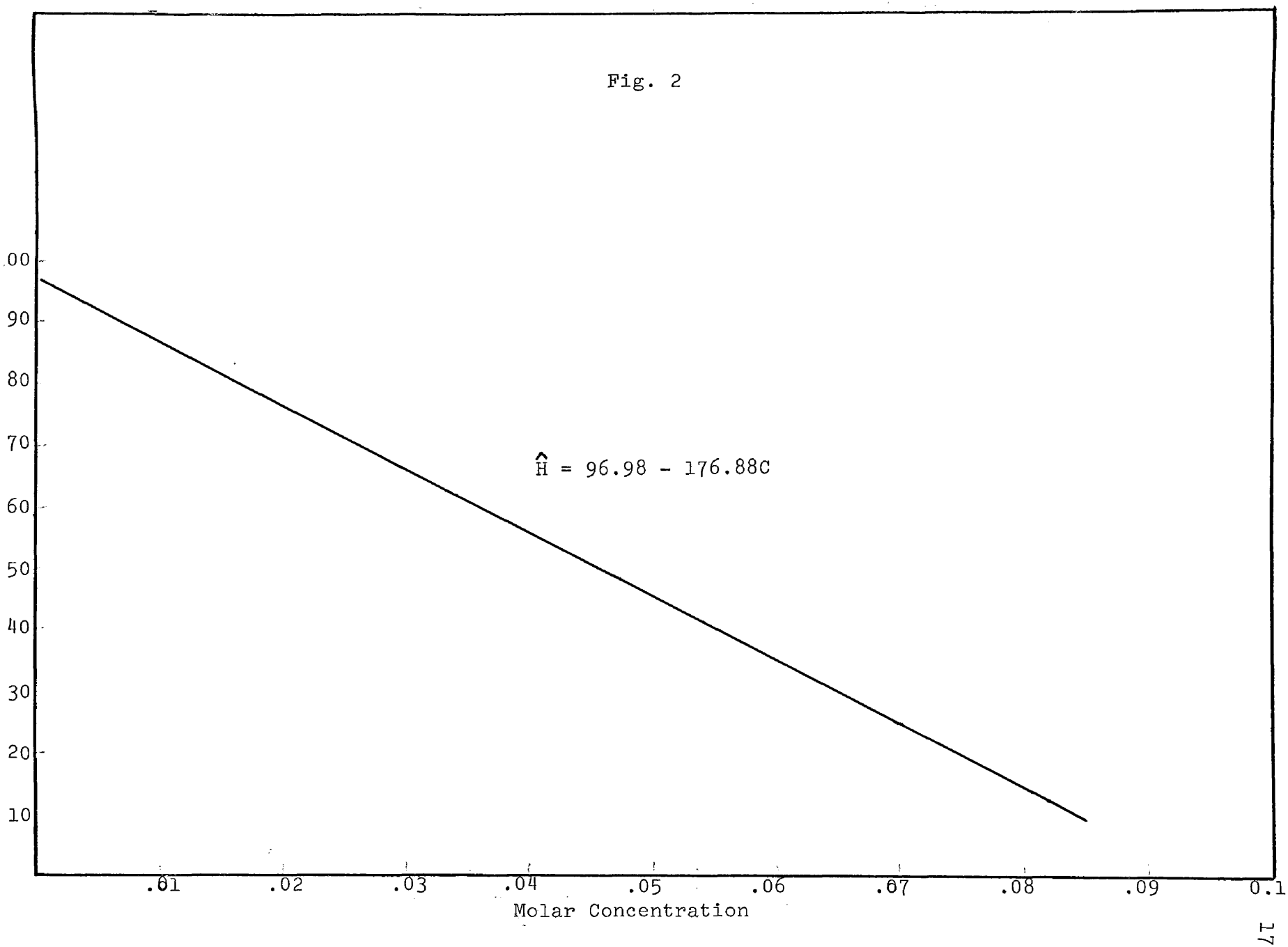

00
90
80
70
60
50
40
30
20
10
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
Molar Concentration

Fig. 2


00
90
80
70
60
50
40
30
20
10
$\hat{H}$ = 96.98 - 176.88C
.01
.02
.03
.04
.05
.06
.07
.08
.09
0.1
Molar Concentration

Vita

Thomas Joseph Andary was born in Sault Ste. Marie, Michigan, on October 8, 1942. He attended parochial schools in the same city, where he was graduated from Loretto Catholic Central High School in 1960. He attended Lake Superior State College from 1960 to 1962, and Northern Michigan University. Majoring in Predentistry he received the B.A. degree in Chemistry. In 1964 he entered The University of Detroit Dental School until 1966 when he left, deciding to change his major area. In 1966 he enrolled in the graduate school of Northern Michigan University, receiving the M.A. in Biology degree in 1968.