# *A Method for Investigating the Hæmolytic Activity of Chemical Substances.*

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## *Introduction.*

Arrhenius has pointed out that the phenomenon of hæmolysis may be described by the qt. rule.* In this investigation it has been found that this rule is quite insufficient to describe the relation between the quantity of a hæmolytic agent and the time which such quantity takes to produce hæmolysis, unless a large margin of error be admitted. Since in some of the examples quoted by Arrhenius, the end point was not complete hæmolysis, but a certain percentage, it is easy to see that the difficulties of investigation account for the fact that the discrepancy between results given by experiment and those obtained by the use of the qt. rule has been overlooked. Further it is recognised that this rule is not of general application; being inapplicable, for instance, in the case of sodium oleate. In order to remove the difficulty of investigation, a technique of great accuracy has been introduced; the results of experiments will be found to require a description other than that furnished by the qt. rule.

## *Special Apparatus Required.*

(1) A set of pipettes to deliver 1, 2, 3, 4 and 5 c.c.

(2) Pipettes, 1 c.c. capacity, graduated in 0·01 of a cubic centimetre.

(3) Burettes and pipettes of convenient capacity, and tared flasks of 50 and 100 c.c. capacity.

All volumetric apparatus must be certified, or preferably calibrated by the experimenter.

(4) Tubes, about $1 \times 3$ inches, with flat bottoms for preference; these have rubber stoppers, with one perforation for the passage of a thermometer.

(5) About twelve thermometers, graduated from 0° C. to 50° C. and capable of being read to 1/10 of a degree: these must all be checked against a standard instrument. The bulbs should be small, and the scale arranged so that it shall be above the level of the stopper of the tube when the thermometer is in position.

* Arrhenius, S., 'Quantitative Laws in Biological Chemistry,' 1915, p. 63, *seq.*

(6) Three water baths, for maintaining temperatures at about 3° C., 12° C. and 30°—50° C. respectively. These should each be capable of containing about six of the tubes above mentioned in such a way that the degree of hæmolysis in the contents of the tubes may be seen without removing the tubes from the bath. The baths are arranged so that the contents of the tubes are seen against a screen lit by artificial light.

*Preparation of the Standard Blood Suspension.*

The suspension used in all the experiments is of an arbitrary, but convenient strength.

It is prepared as follows:—0·5 c.c. of blood is drawn from the finger; the blood must flow easily, and the finger must not be squeezed. This volume of blood is added to a tube containing 15 c.c. of 1·5 per cent. citrated saline; the receiving pipette should be rinsed with this saline, and drained, before drawing up the blood. The contents of the tube are then centrifuged, the clear supernatant fluid removed, and the tube again filled, this time with 0·95 per cent. sodium chloride. Again it is centrifuged. This process is repeated, so as to make four washings in all. After the final washing, the supernatant saline is removed, the tubes put in the centrifuge for a few minutes, and the last drops of saline taken off the cells with a capillary pipette. The cells are then added to 10 c.c. of 0·95 per cent. sodium chloride.

The resulting blood suspension will keep for fully eighteen hours, but should be used soon after preparation. In carrying out series of experiments, it is important that the blood used should be derived from the same person, *e.g.*, from the experimenter. During more than a year's work, involving the preparation of nearly a thousand such suspensions, and using the blood of the same person, a variation of the suspension sufficiently great to be detected has never occurred. It may, therefore, be taken that the suspension, if carefully prepared, is reliably constant in strength; it is, however, most necessary that the cells be freed from all traces of serum.

All the formulæ given refer to this suspension: the modifications of the formulæ required if suspensions of other strength are used will be given later.

*Technique.*

To investigate the hæmolytic activity of a hæmolytic substance, the following technique is employed. Since the activity of such a substance depends on (1) the dilution of the substance, (2) the quantity of blood suspension which it has to hæmolyse, (3) the time which it takes to complete this hæmolysis, and (4) the temperature at which the experiment is conducted,

each of these variables must be controlled or measured: where a standard blood suspension is used, (2) may be neglected.

1. A solution of convenient strength in 0·95 per cent. NaCl of the hæmolytic substance to be investigated is prepared: as a rule, a solution 4 c.c. of which contains 50 mgrm. of the substance is employed. In the case of the bile salts, a solution of about this strength is essential; it is further very convenient to have the solution so adjusted, as it facilitates calculation of dilutions. From this solution dilutions are made as required. A series of dilutions are made, *e.g.*, 1·1000, 1·2000, 1·3000, 1·4000. 4 c.c. of each dilution is placed in a series of tubes with thermometers, as described above. The tubes are then placed in the water bath at, say, 40° C.; with them is placed a tube containing blood suspension. When the temperature of each tube has reached the temperature of the bath, as indicated by the thermometers carried by each, the blood suspension is added and the experiment commenced. This requires care and practice to perform satisfactorily and is done as follows: The tube containing the blood suspension is inverted rapidly, to mix the contents, and is then replaced in the bath. The pipette of 1 cc. capacity with which the measured quantity of blood suspension is to be added to each tube, is warmed by drawing up through it saline which has been warmed to a moderate heat over a flame. It is then at once dipped into the tube containing the blood suspension, of which 1 c.c. is drawn up; this is delivered without delay into the tube containing the strongest solution of hæmolytic agent (in the above series the 1·1000 tube). The time is noted; the process of adding blood suspension is repeated in the case of each of the tubes containing dilutions of hæmolytic agent, the moment of adding the blood being observed in each case. A stop-watch is almost essential. The operation requires to be carried out as quickly as possible. There should be scarcely an appreciable loss of temperature in the contents of the tubes when the blood is added. This result can be attained by speed and practice; although the method may appear clumsy it is open to fewer objections than is the method of adding the suspension by upsetting a small tube in which it is contained, and which is placed inside the large tube. It is possible to carry out this operation for ten tubes within thirty seconds, and with a loss of no more than 1/10° C. From the moment of adding the suspension the temperature in each tube should remain constant.

The moment of complete hæmolysis in the case of any tube is decided preferably by comparison with a fully hæmolysed control tube, which is placed in the bath. The water bath being placed against an illuminated screen, it is easy to compare the intensity of blackness with which a black rod placed horizontally across the screen is seen, in the case of tube and

control respectively. This method of deciding the end-point is very satisfactory, as, if the tube be inspected by transmitted light alone, without a dark background, the time taken for complete hæmolysis will be much underestimated. The moment when complete hæmolysis occurs is noted; the time taken for the particular dilution of hæmolytic agent to hæmolyse 1 c.c. of standard blood suspension at the particular temperature employed is then known.

If the time for hæmolysis is long, the contents of the tubes should be stirred with the thermometer each half-hour.

If 4 c.c. of the dilution of hæmolytic agent contain 5 mgrm. (that is, a 1·10 dilution of the original solution prepared), then, when 1 c.c. of the blood suspension is added, the 5 c.c. in the tube will contain 5 mgrm. of hæmolytic agent, or a 1·1000 dilution (ignoring the negligible volume of the erythrocytes). In this way the dilution employed for producing hæmolysis is kept in round figures and readily calculable.

This method of investigating hæmolytic activity has been applied to a number of hæmolytic substances. Those investigated were the following:—

| | |
|---|---|
| Saponin. | Acetic acid. |
| Sodium taurocholate. | Citric acid. |
| Sodium glycocholate. | Benzoic acid. |
| Lactic acid. | Ammonium chloride. |

It has been found possible to describe the action of these substances by formulæ.

It is to be remembered that, while the general forms of these formulæ are correct for the action of these substances on blood suspensions of any concentration, the values of the various constants in the equations will vary for blood suspensions of different concentrations, and even for suspensions of the same concentration, of blood cells derived from different individuals or animals.

*Relation between Time and Temperature.*

In the cases of all hæmolytic substances examined, it has been found by experiment that there is a definite relation between the time taken for a given quantity of the substance to produce complete hæmolysis of 1 c.c. of standard blood suspension, and the temperature at which the experiment is conducted. As the temperature is increased, the time taken to produce complete hæmolysis becomes less.

If T = the time, in minutes, taken to produce hæmolysis of 1 c.c. standard blood suspension,

and $\tau$ = the temperature at which the experiment is conducted, in degrees centigrade,

the relation between T and $\tau$ is expressed by a hyperbola, of which one asymptote is the straight line T = 0. The relation is therefore described by the equation

$$\tau = \frac{\alpha}{\beta}[\beta - T] + \frac{\gamma}{T}.$$

where $\alpha$, $\beta$, and $\gamma$ are constants which depend on the quantity of hæmolytic substance whose action the hyperbola describes.

In order to find by experiment, within the limits of experimental error, the values of $\alpha$, $\beta$, and $\gamma$ for a given quantity of hæmolytic substance, the following procedure is used:—

(1) Find by experiment the time taken by this quantity of hæmolytic substance to produce hæmolysis of 1 c.c. of standard blood suspension, when $\tau_0$ = any value near 0. Call this time $T_0$.

(2) In a similar manner find $T_1$, when $\tau_1$ = any value near 10.

(3) Similarly find $T_2$, when $\tau_2$ = any value between 30 and 45.

(4) Treat that part of the hyperbola which describes the relation between T and $\tau$, when $\tau$ is any value from 0 to 10, as a straight line, which will pass through the points $T_0$ and $T_1$: determine the intercepts made by this line on the axes of T and $\tau$ respectively. The intercept on the T axis will equal $\beta$; that on the $\tau$ axis will equal $\alpha$.

(5) Filling in the values of $\alpha$ and $\beta$, obtained as above (4), and the values of $T_2$ and $\tau_2$ obtained experimentally in (3), in the equation

$$\tau_2 = \frac{\alpha}{\beta}(\beta - T_2) + \frac{\gamma}{T_2},$$

find the value of $\gamma$ for the hyperbola describing the relation between T and $\tau$, for the particular quantity of hæmolytic substance under investigation.

The equation expressing the relation between T and $\tau$ for any given quantity of hæmolytic substance may thus be found from three experiments. The values of $\alpha$, $\beta$ and $\gamma$ obtained should be checked by comparing experimental and calculated values of T, when $\tau = 20$, $\tau = 30$, $\tau = 40$, and $\tau = 50$. If $\alpha$ and $\beta$ are accurately determined, an excellent correspondence between calculated and experimental results will be obtained.

In investigating the hæmolytic action of a substance, the equations for hyperbolas representing the relation between T and $\tau$, in the case of each of several dilutions of the substance, are obtained. A series of values of $\alpha$, $\beta$, and $\gamma$ are thus found, corresponding to various dilutions: these constants may then be expressed as functions of the dilution.

*General Equations relating $\alpha$, $\beta$, and $\gamma$ to the Dilution.*

In the cases of all the hæmolytic substances examined, these constants are related to the dilution in a similar way :—

(1) The constant $\alpha$ is a linear function of the dilution. If $\delta$ be the number of cubic centimetres which contains 1 grm. of the hæmolytic agent, in the dilution investigated, then the relation between $\alpha$ and $\delta$ is expressed by an equation of the form

$$\alpha = m\delta + n,$$

where $m$ and $n$ are constant for the particular hæmolytic substance.

(2) The constant $\beta$ is related to $\delta$ by a curve. If on the curve points, $p_1$, $p_2$, $p_3$, etc., corresponding to values, $\delta_1$, $\delta_2$, $\delta_3$, etc., be joined to the origin $\beta = 0$, $\delta = 0$, a series of angles $\theta_1$, $\theta_2$, $\theta_3$, etc., will be formed between the joining lines and the abscissa. These angles, expressed in degrees, are related to $\delta_1$, $\delta_2$, $\delta_3$, etc., by a hyperbola : the relation being expressed by the equation

$$0{\cdot}01\,\delta = \frac{a}{b}[b-\theta] + \frac{c}{\theta},$$

since the hyperbola has one asymptote, the straight line $\theta = 0$. The relation between $\beta$ and $\delta$ is therefore expressed by the equations

$$0{\cdot}01\,\delta = a - \frac{a\theta}{b} + \frac{c}{\theta}, \qquad \text{(i)}$$

$$0{\cdot}01\,\delta = \beta \tan\theta, \qquad \text{(ii)}$$

where $a$, $b$ and $c$ are constant for the particular hæmolytic substance.

(3) The relation between $\gamma$ and $\delta$ is expressed by an equation of the form

$$\gamma = p\delta,$$

where $p$ is a constant for the particular hæmolytic substance. Since $\alpha$, $\beta$, and $\gamma$ are related to the dilution in these ways, by a knowledge of the equations given above, and of the values of $a$, $b$, $c$, $m$, $n$, and $p$, for a hæmolytic substance, it is possible to calculate the time taken by any dilution of that substance to produce complete hæmolysis of 1 c.c. of standard blood suspension, at any given temperature, or to make other calculations involving these variables.

In order to illustrate these general relations, the results obtained from an examination of the hæmolytic action of three substances will be given in detail. The substances selected—saponin, sodium taurocholate, and lactic acid—are chosen because they are examples of a highly hæmolytic agent, a moderately hæmolytic agent, and a feebly hæmolytic agent respectively :—

1. *Saponin.*

The following values were obtained for the constants in the equations relating to T and $\tau$:—

| $\delta$. | $\alpha$. | $\beta$. | $\gamma$. |
|---|---|---|---|
| 5000 | 9·4 | 25 | 4·1 |
| 10000 | 10·4 | 57 | 8·3 |
| 20000 | 12·4 | 139 | 16·6 |
| 30000 | 14·4 | 252 | 25 |

From these, the following values are calculated for the constants in the equation relating $\alpha$, $\beta$ and $\gamma$ to $\delta$:—

| $a$. | $b$. | $c$. | $m$. | $n$. | $p$. |
|---|---|---|---|---|---|
| 1300 | 65 | 0·4 | 0·0002 | 8·4 | 0·00083 |

The correspondence between observed and calculated results is shown by the following Tables:—

$\delta = 5{,}000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 10 | 2·3 | 2·5 |
| 30 | 0·17 | 0·2 |

$\delta = 20{,}000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 12 | 16 | 15 |
| 40 | 0·4 | 0·5 |

$\delta = 50{,}000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 40 | 2·3 | 2 |

2. *Sodium Taurocholate.*

Certain points have to be noted regarding this salt.

(1) A pure dry specimen must be obtained. Dryness is ensured by desiccating *in vacuo* for about a fortnight.

(2) Sodium taurocholate does not form a true solution. In saline, water, or dilute alcohol, it becomes opalescent, and when in this state will all pass through a fine filter. Its behaviour is similar to that of a soap. The rapidity with which this opalescence forms depends on the dilution, a 1 per cent. solution remaining clear for some time, while a 0·1 per cent. solution becomes rapidly cloudy. The stability is lessened by heating. Consequently it is necessary (*a*) to make the original solution, from which dilutions are to be made, not more dilute than 1 per cent.; (*b*) to make the dilutions and perform the experiments as rapidly as possible; and (*c*) to avoid heating the solution unnecessarily.

The following values were obtained for the constants in the equations relating T and $\tau$:—

| $\delta$. | $\alpha$. | $\beta$. | $\gamma$. |
|---|---|---|---|
| 1000 | 15 | 9 | 20 |
| 2000 | 19 | 31 | 40 |
| 2500 | 21 | 57 | 50 |
| 3000 | 23 | 100 | 60 |
| 3500 | 25 | 200 | 70 |
| 4000 | 27 | 570 | 80 |

From these are derived the following values for the constants in the equations relating $\alpha$, $\beta$, and $\gamma$ to $\delta$:—

| $a$. | $b$. | $c$. | $m$. | $n$. | $p$. |
|---|---|---|---|---|---|
| 40 | 65 | 10 | 0·004 | 11 | 0·02 |

The correspondence between calculated and experimental results is shown by the following tables:—

$\delta = 1000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 10 | 5 | 5·3 |
| 30 | 1 | 1·2 |

$\delta = 2000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 20 | 7 | 7·3 |
| 30 | 3 | 3 |

$\delta = 3000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 20 | 60 | 60·7 |
| 40 | 3·5 | 3·4 |

$\delta = 4000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 12 | 330 | 324 |
| 40 | 6 | 6·3 |

$\delta = 7000$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 45 | 22 | 23·1 |

### 3. *Lactic Acid.*

This is a feebly hæmolytic agent. The end point is difficult to determine, especially at high temperatures, owing to colour changes produced in the hæmoglobin. The following values were obtained for the constants in the equations relating T and $\tau$:—

| $\delta$. | $\alpha$. | $\beta$. | $\gamma$. |
|---|---|---|---|
| 1000 | 26·6 | 64 | 40 |
| 1250 | 29·1 | 152 | 50 |
| 1666 | 33·2 | 389 | 66 |
| 1785 | 34·4 | 477 | 71 |
| 2000 | 36·6 | 636 | 80 |

From these are derived the following values for the constants in the equations relating $\alpha$, $\beta$ and $\gamma$ to $\delta$:—

| $a$. | $b$. | $c$. | $m$. | $n$. | $p$. |
|---|---|---|---|---|---|
| 9 | 65 | 20 | 0·01 | 16·6 | 0·04 |

The following tables compare calculated and experimental results:—

| $\delta = 1000$. | | | $\delta = 2000$. | | |
|---|---|---|---|---|---|
| $\tau$. | $T_{exper.}$ | $T_{calc.}$ | $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
| 10 | 42 | 42·3 | 30 | 125 | 127 |
| 30 | 10·5 | 10·6 | 40 | 47 | 48 |

The general relations between T, $\tau$ and $\delta$, for any of the hæmolytic substances examined, having been dealt with, there remains to be expressed certain relations which exist between the constants of the equations relating $\alpha$, $\beta$ and $\gamma$ to the dilution. These relations hold true for all the substances examined, and probably for most hæmolytic substances.

It will be seen that the hæmolytic activity of a substance depends principally on the manner in which $\alpha$ and $\beta$ vary with the dilution. The variation of $\alpha$ with the dilution depends chiefly on the value of $m$, while the variation of $\beta$ with dilution depends chiefly on the value of $a$. There must, then, be a relation between the value of $n$ and the value of $a$, for any substance.

Let $$a/b = \tan \phi,$$

and $$100m = \tan \omega,$$

the relations between $\phi$ and $\omega$ is expressed by a hyperbola, whose equation is

$$0{\cdot}75\omega^2 + \omega(\phi - 45) - 52 = 0.$$

Since $b$ is a constant for all hæmolytic substances examined (65), from a known value of $a$ the corresponding value of $m$ can be found.

The relation between $\alpha$ and $\delta$ further depends on the value of the constant $n$. This constant is related to the value of $m$. If, as above,

$$100m = \tan \omega,$$

the relation between $\omega$ and $n$ is expressed by a rectangular hyperbola, and by the equation,

$$n(90 - \omega) = 750.$$

The relation of $\beta$ to the dilution depends to some extent on the value of $c$; this constant has a simple relation to the constant $p$,

$$c = 500p.$$

The value of $c$ may be readily found for any substance, for which one value of $\gamma$ is known.

From a knowledge of these general relations, if three suitable experimental readings be given for a substance, the time taken by it to produce hæmolysis under any circumstances of temperature or dilution may be calculated.

As an example:—Using acetic acid (S.G. = 1·044), it is found that the following Table gives the relation between certain values of T and $\tau$, when $\delta = 1000$,

| $\tau$. | T. |
|---|---|
| 2 | 135 |
| 7 | 100 |
| 44·1 | 4 |

From these values may be found,

$$\alpha = 36{\cdot}4$$
$$\beta = 143$$
$$\gamma = 40$$

From the value of $\gamma$ we find first $p$ and then $c$. From the values of $\beta$ and $\delta$ the value of $\theta$ in the equation relating $\delta$ and $\beta$ may be found. Since $c$ is known, the value of $a$ in this equation may be calculated, $b$ being taken as 65. When $a$ and $b$ are known, $\phi$ may be calculated and from it the value of $\omega$. This value gives the values of $m$ and $n$. Thus, we may find for acetic acid that the following values are true:—

| $a$. | $b$. | $c$. | $m$. | $n$. | $p$. |
|---|---|---|---|---|---|
| 5 | 65 | 20 | 0·0146 | 21·8 | 0·04 |

The constants being known, the values of $\alpha$, $\beta$, and $\gamma$ for any dilution may be calculated. For instance, when $\delta = 1785$,

| $\alpha$. | $\beta$. | $\gamma$. |
|---|---|---|
| 37·8 | 1136 | 71 |

The value of T corresponding to any value of $\tau$ for this dilution may be found in the usual way; for example,

$\delta = 1785$.

| $\tau$. | $T_{exper.}$ | $T_{calc.}$ |
|---|---|---|
| 32 | 185 | 188·7 |

While it is thus possible to calculate with considerable correctness the relation of T and $\tau$ for any dilution of a substance for which only three experimental readings are given for one dilution, it is advisable to take three readings for each of several dilutions, the accuracy of the determination of the constants $a$, $b$, $c$, $m$, $n$, and $p$ being greatly increased thereby. If a sufficient number of experimental readings for various dilutions are available, results may be obtained graphically.

*Summary.*

1. A technique for the investigation of the hæmolytic action of chemical substances is described.

2. The relation between the time taken by a given quantity of hæmolytic substance, and the temperature at which it acts, is expressed by a hyperbola.

3. Equations are given expressing the relation between the constants of such a hyperbola and the quantity of hæmolytic substance to which the hyperbola applies.

4. Certain general relations, which have been found to hold for all substances examined in connection with this research, are pointed out.

5. A comparison between experimental and calculated results is given.

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# *Observations on the Effects of Fat Excess on the Growth and Metamorphosis of Tadpoles.*

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(Communicated by Prof. C. S. Sherrington, Pres.R.S. Received June 10, 1921.)

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Previous experimentation* has shown that in certain circumstances the presence of excessive amounts of fat in the food of animals may be harmful. Thus, an excess of butter in association with a dietary of autoclaved rice hastens the death of both pigeons and monkeys, and gives rise to changes in the internal organs more pronounced than those resulting from an autoclaved rice dietary alone. Again, an excess of butter in association with a dietary of mixed grains and peas causes enlargement, with hyperplasia and vesicular budding, of the thyroid gland in pigeons, identical with that characteristic of Graves' disease.† This enlargement of the thyroid gland is associated with a reduction in size of the adrenal glands. If, however, fresh onions be added to the dietary of mixed grains and butter both the incidence of the thyroid enlargement and the intensity of the hyperplasia are reduced; while the associated diminution in size of the adrenal glands is not so marked.

This observation as to the effect of an otherwise adequate food containing an excess of butter in producing thyroid hyperplasia of the Graves' disease

* McCarrison, R., 'Ind. Jour. Med. Res.,' vol. 6 (4), p. 550, and vol. 7 (2), p. 308 (1919).

† McCarrison, R., 'Ind. Jour. Med. Res.,' vol. 7 (3), p. 633 (1920).