CLIII. THE DESTRUCTIVE ACTION OF HEAT ON INSULIN SOLUTIONS.

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WE have endeavoured to determine the relationship between temperature and destruction of sterile watery buffered solutions of insulin (about 15 international units per mg.) and to find a mathematical expression for it. According to the law of mass action we might expect that the rate at which insulin is destroyed at a constant temperature at any moment should be proportional to its concentration as expressed by the well-known equation

$$k = \frac{1}{t} \cdot \log \frac{a}{a-x}$$

where k is the velocity constant, a the initial concentration, and x the amount destroyed at the time t . This expression can be used to evaluate k , the quantity $(a - x)$ left at a given time t being estimated by biological standardisation.

Experiments were carried out at 50°, 60°, 80°, 100° and 117°.

The constant temperatures were obtained by using an electrical incubator (at 50°, 60° and 80°), boiling water or vapour from boiling water (100°) and vapour from boiling normal primary butyl alcohol (117°).

For the experiments at 50°, 60°, 80° and 100° the solution was placed in rubber-capped vials, whereas at 117° we used test-tubes (Jena Fiolax glass) sealed in the blow-pipe. They were placed at the constant temperatures and after the lapse of a certain period of time they were taken out and instantly cooled in water. The potency of each sample was compared with the potency of a sample of the same solution which had been left in the ice-chest, where no measurable destruction takes place within very long periods of time. The constants evaluated from the experiments are given in Table I.

In order to decide whether or not the equation employed holds good for the destruction process we have made experiments at 60° (one experiment), 100° (three experiments), and 117° (two experiments) in which samples (each in a separate vial) were taken out at various periods of time. By placing a thermometer in one of the vials used at 100° and a metallic alloy melting just below 117° in one of the tubes used at that temperature it was found that the time elapsing before the liquid in the tubes became heated to the surrounding temperature could have no appreciable influence on the result (*i.e.* the error

of the time of heating would not exceed 1 % even in the shortest experiment). The results of the experiments are given in Table I.

Table I.

Temp. ° C.	<i>t</i> (hours)	Initial p_H *	$\frac{a-x}{a}$	<i>k</i>	<i>a</i> (units per cc.)	Brand†	Test†	No. in Fig. 2
50.1	623.0	4.5	0.66	0.000296	20	Leo	r.	1
50.1	623.0	3.5	0.60	0.000360	15	Leo	r.	2
60.0	192.0	4.0	0.52	0.00147	20	Leo	m.	3
60.0	360.0	4.0	0.39	0.00113	20	Leo	m.	4
60.0	696.0	4.0	0.116	0.00134	20	Leo	m.	—
60.0	1030.0	4.0	0.076	0.00109	20	Leo	m.	—
60.0	143.0	4.5	0.77	0.000797	—	Leo	r.	5
60.3	181.0	3.5	0.59	0.00128	—	Leo	r.	6
60.0	308.5	—	0.47	0.00107	—	Leo	r.	7
80.0	16.0	—	0.82	0.00537	—	B.D.H.	r.	—
79.7	16.2	3.5	0.52	0.0178	—	Leo	r.	8
81.0	20.0	—	0.36	0.0219	—	Leo	r.	9
100.0	1.0	4.0	0.74	0.131	20	Leo	m.	—
100.0	2.0	4.0	0.43	0.183	20	Leo	m.	—
100.0	3.08	4.0	0.425	0.121	20	Leo	m.	—
100.0	4.0	4.0	0.29	0.134	20	Leo	m.	—
100.0	6.0	4.0	0.35	0.076	20	Leo	m.	—
100.0	8.0	4.0	0.38	0.053	20	Leo	m.	—
100.0	2.5	4.0	0.475	0.129	20	Leo	m.	10
100.0	8.0	4.0	0.195	0.089	20	Leo	m.	—
100.0	1.73	4.0	0.50	0.174	40	Leo	m.	11
100.0	3.5	4.0	0.27	0.163	40	Leo	m.	—
100.0	5.26	4.0	0.228	0.122	40	Leo	m.	—
100.0	7.0	4.0	0.125	0.129	40	Leo	m.	—
100.0	9.3	4.0	0.074	0.121	40	Leo	m.	—
100.0	10.53	4.0	0.046	0.127	40	Leo	m.	—
100.0	2.0	—	0.50	0.150	—	B.D.H.	r.	12
100.0	2.0	4.0	0.57	0.124	—	Leo	r.	13
100.0	2.0	3.5	0.60	0.111	—	B.W.	r.	14
117.0	0.542	4.0	0.415	0.705	20	Leo	m.	15
117.0	1.04	4.0	0.355	0.432	20	Leo	m.	—
117.0	2.21	4.0	0.177	0.34	20	Leo	m.	—
117.0	2.73	4.0	0.135	0.319	20	Leo	m.	—
117.5	1.0	4.0	0.332	0.478	40	Leo	m.	16
117.5	2.05	4.0	0.18	0.362	40	Leo	m.	—
117.5	3.0	4.0	0.10	0.33	40	Leo	m.	—
117.5	4.0	4.0	0.048	0.33	40	Leo	m.	—
117.5	5.0	4.0	0.029	0.308	40	Leo	m.	—

* At the time when the experiments were made the colorimetric method of p_H determination was used (bromophenol blue). When later on the hydrogen electrode was employed it turned out that a considerable correction had to be made. Thus in reality the values of $p_H=4$ in the table correspond to $p_H=3.2$ as measured by the hydrogen electrode.

† Abbreviations: B.D.H., British Drug Houses; B.W., Burroughs Wellcome; r., rabbit test; m., mouse test.

From the equation quoted above the logarithm of undestroyed insulin ($a-x$) can be expressed as a linear function of heating time, t ,

$$\log(a-x) = -kt + \log a.$$

Since a is the initial concentration, $\log a$ is constant, and accordingly the results obtained from the experiments at 60°, 100° and 117° should be represented by a straight line when plotting heating time against the logarithm of undestroyed insulin. In Fig. 1 this is illustrated in the case of one of the experiments (at 117.5°). Apparently a line connecting the points would in

some of the experiments have a tendency to bend upwards during its course, indicating that the destruction becomes relatively slower as the activity decreases, but in no cases except perhaps in one experiment at 100° can this be said with certainty. The velocity constant calculated from different points at the same temperature shows variations of 50–100 % (see Table I) probably for the most part owing to experimental errors, but perhaps also to some extent because the equation fails to express quite exactly the mechanism of the process.

However, the variations of the constant calculated for the same temperature are negligible when compared with the difference between the averages of the constants calculated for different temperatures, *e.g.* the constant at

Fig. 1.

100° is a hundred times larger than the constant at 60°, and the constant at 117° is about three times as large as the constant at 100°.

In spite of the rather large dispersion of the constants for each temperature the wide range of temperatures employed appears sufficient as regards the determination of the exact relation of velocity constant to temperature.

Arrhenius's relation between absolute temperature T and the velocity coefficient k can be written in terms of a linear relation

$$\log_{10} k = -\frac{0.43 \cdot \mu}{2} \cdot \frac{1}{T} + \text{constant},$$

and its applicability can be tested by plotting $\log k$ against $1/T$.

As seen in Fig. 2 the process follows closely the law of Arrhenius, at least within the limits of temperatures employed. Only constants evaluated from

nearly the same stage of destruction, namely half destruction, have been employed since this seems to be the most proper way of comparison.

In view of the wide divergence of temperatures examined it seems fairly probable that the relation found will be valid over an even wider range of temperatures. Assuming this, the time taken for the activity to fall 5, 10 and 50 % has been calculated for a series of lower temperatures, at which it is difficult to make experiments owing to the slow rate of destruction. The results are given in Table II.

Fig. 2.

Table II.

	Time taken for the activity to fall		
	5 %	10 %	50 %
40°	$\frac{1}{2}$ month	1 month	$\frac{1}{2}$ year
30°	2 months	4 months	2 years
20°	9 "	1 $\frac{1}{2}$ years	10 "
10°	4 $\frac{1}{2}$ years	8 $\frac{1}{2}$ years	56 "
0°	29 "	58 "	377 "

Since the values given in Table II are extrapolated values, the value for 0° will be much less accurate than those for 30° and 40°. Judging from calculations

based upon displacement of the straight line in Fig. 2 into extreme and improbable situations the accuracy of the values for 0° is 50–100 %, and for 30° about 25 %.

The value of the constant μ calculated from the straight line drawn in Fig. 2 is 28,300, which is a very high value as compared to the values met with in most chemical reactions. Generally μ is between 10,000 and 17,000. High values of μ are by some authors especially ascribed to monomolecular reactions. Whether the apparent change in the relative velocity during a single destruction experiment may be due to the formation of products inhibiting the destruction or to some other reason, must be the aim of future research. We ought to mention that there is invariably a precipitation of inactive material at this p_H during the heating process and that there is a shift of p_H during the destruction process in spite of the buffer. Applying the hydrogen electrode at 18° to a solution before and after 2 hours' heating at 100° we found a shift of p_H from 3.24 to 3.40. As standard electrode the Veibel quinhydrone electrode was used [Biilmann, 1925].

The influence of temperature on the buffer action of the solutions employed was tried by placing the same solution at 18° and at 70° (in a water-bath covered with oil) and applying a hydrogen electrode. As a standard electrode at 70° another hydrogen electrode was used in 0.01 *N* HCl + 0.09 *N* KCl which was supposed to have the same p_H at 18° and 70° . Owing to the high vapour pressure at 70° the experiment could not be of long duration. A constant potential was rapidly obtained corresponding to $p_H = 3.31$. The same solution showed $p_H = 3.24$ at 18° . Applying to another solution the hydrogen electrode at 18° , 25° and 37° (in water thermostats), using the Veibel quinhydrone electrode as a standard electrode and applying its temperature coefficient as quoted by Biilmann [1925], the shift of p_H was found to be 0.01. We conclude from these experiments that there is no large shift of p_H caused by temperature or taking place in the course of a destruction experiment. But as we know nothing exactly about the influence of p_H on the rate of destruction the shifts of p_H might to some extent be the cause of the dispersion of the constants.

For the purpose of determining the influence of shifts of p_H on the destruction rate a number of solutions were colorimetrically adjusted to different values of p_H and supplied with buffers, and the p_H values were checked by hydrogen electrode measurements. The solutions adjusted to p_H about 4 and 6.5 have in most cases been slightly opalescent.

The buffers added were such as have no temperature coefficient according to Walbum [1920], at least between 18° and 70° , namely the citrate-HCl mixtures and the phosphate mixtures of Sørensen [1909]. The influence of temperature on the buffer action of insulin itself is very slight according to the experiments quoted above (when the buffer added was also one that had no temperature coefficient between 18° and 70°).

As seen in Table III the colorimetric method and the electrometric method do not agree when bromophenol blue and bromocresol green are used as

indicators. All the electrometrically obtained values given are calculated from potentials which had attained a constant value (*i.e.* constant within about half a millivolt or less in the course of a quarter of an hour). For the calculation of the values of p_H 1-3 the Bjerrum [1911] extrapolation formula has been applied in order to eliminate the liquid junction potential, first using 3.5 *N* KCl and then using 1.75 *N* KCl to connect the two electrodes. In the other cases only 3.5 *N* KCl was used. For all the measurements one of two platinum electrodes was used which had been shown to produce the same potential when used in the same solution.

Table III.

Batch	p_H		Buffer (i)	t (hours)	a	$a - x$	k	Temp. ° C.	
	Color.	Electr.							
A	1.2	(a)	1.10	1.0	0.75	18.7	11.9	0.261 (g)	100.8
B	1.2	(a)	1.17	1.0	1.0	20.0	11.0	0.26 (g)	100.2
B	1.2	(a)	1.17	1.0	1.225	20.0	9.9	0.249 (h)	100.6
C	2.0 ± 0.1	(a)	1.73	0.25	1.975	20.0	11.1	0.130 (h)	100.1
D	2.0 ± 0.1	(a)	1.79	0.25	1.975	20.0	11.5	0.122 (h)	100.1
E	1.9	(a)	1.78	0.25	4.0	20.0	5.1	0.149 (g)	100.4
A	1.9	(a)	1.76	0.50	2.0	13.3	8.1	0.108 (g)	100.0
C	2.45 ± 0.15	(a)	2.42	0.25	1.975	20.0	10.9	0.134 (h)	100.1
D	2.45 ± 0.15	(a)	2.34	0.25	1.975	20.0	11.8	0.116 (h)	100.1
E	2.8 ± 0.5	(a), (b)	—	0.25	3.987	20.0	8.6	0.092 (g)	100.4
A	3.0	(a), (b)	2.88	0.57	2.0	11.0	6.1	0.128 (g)	100.0
C	4.2 ± 0.2	(c)	3.63	0.25	1.975	20.0	10.8	0.135 (h)	100.1
D	4.2 ± 0.2	(c)	3.65	0.25	1.975	20.0	9.8	0.157 (h)	100.1
E	4.0	(d)	3.58	0.25	3.975	20.0	7.2	0.112 (g)	100.4
C	6.5 ± 0.2	(d)	6.75 (f)	0.25	0.975	20.0	7.4	0.432 (h)	100.6
C	6.5 ± 0.2	(d)	6.75 (f)	0.025	0.975	20.0	10.3	0.289 (h)	100.6
B	7.45 ± 0.3	(e)	7.43	0.80	0.0833	20.0	12.7	2.36 (h)	100.2

(a) thymol blue; (b) bromophenol blue; (c) bromocresol green; (d) bromophenol blue; (e) cresol red; (f) in reality this solution was not electrometrically checked: three other different solutions prepared in a similar way were colorimetrically adjusted to $p_H = 6.5 \pm 0.2$ and showed $p_H = 6.81$, 6.75 and 6.70 with the hydrogen electrode; (g) sealed test-tubes; (h) rubber-capped vials; (i) buffer content expressed in proportion to buffer content of Sørensen's standards of the same p_H .

The solutions were submitted to a temperature of 100° for a period of time supposed to cause half destruction and then compared with a sample of the same solution which had been submitted to 100° for 1.5 minutes. Since this is the time taken for the vials to reach 100° the error of a too low constant in short experiments will be avoided provided that 1.5 minutes is subtracted from the apparent heating time when evaluating the constant.

The results of these experiments are given in Table III and Fig. 3. It is seen from the figure that the influence of the slight p_H variations in the destruction experiments is too small to account for the dispersion of the constants in either Fig. 2 or 3, when p_H is about 3. For the purpose of comparing the dispersion of the constants in Figs. 2 and 3 the logarithms of the constants have been chosen as ordinates also in Fig. 3, and the results from the experiments at 100° in Fig. 2 have been inserted at p_H 3.2 in Fig. 3.

For the purpose of mutual control some of the estimations of insulin activity have been performed by the rabbit blood-sugar test as described by Marks [1926] and others by the mouse convulsion test as described by Krogh

and Hemmingsen [1926]. In Fig. 2 the points obtained from the mouse method (marked ●) show good agreement with the points obtained by the rabbit method (marked ×).

A closer study reveals that the dispersion of the constants for each temperature is larger than might be expected from the accuracy usually obtained with the methods of assay under consideration. This must be ascribed to unknown sources of error in the destruction experiments. It ought also to be mentioned that single determinations of partly destroyed samples show a tendency to larger dispersion than do undestroyed samples, perhaps because of different conditions of resorption in the animals.

Fig. 3.

SUMMARY.

1. The destruction of insulin at a constant temperature is found to follow fairly well the law of monomolecular reactions, the rate being at any moment proportional to the concentration.
2. The relation of rate of destruction to temperature can be well expressed by the formula of Arrhenius. The critical thermal increment amounts to 28,300.
3. The rate of destruction is not appreciably altered by shifts of p_H when this is between 2 and 4.

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