XXXIX. THE CHEMICAL NATURE OF A BACTERIAL HAEMOLYSIN.

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I. THE RELATION BETWEEN THE VELOCITY OF HEAT-DESTRUCTION OF A BACTERIAL HAEMOLYSIN AND THE TRUE REACTION OF THE SUBSTRATE.

Our present knowledge of the effect of heat, and also of acid and alkali on the velocity of destruction of bacterial haemolysins, is due to Famulener and Madsen [1908]. They were able to show that in all cases, whether by altering the temperature or the degree of acidity or alkalinity of the medium, the process of destruction proceeded as a reaction of the first order—that is to say, the destruction at any instant was proportional to the amount of haemolysin then present. They found that as they increased the quantity of acid or alkali, the velocity constant increased and there was a minimum velocity somewhere in the neighbourhood of the neutral point. The exact relationship of this minimum to neutral reaction of the medium, they did not determine however, as the hydrogen ion concentration was not measured. Since that time the technique of the determination of the hydrogen ion concentration of biological liquids by the electrical method has greatly developed and at the present day we can find its value with a very considerable degree of accuracy. It is therefore interesting to investigate how the velocity of destruction varies with the hydrogen and hydroxyl ion concentration. The fact that the velocity increases both with acidity and alkalinity is strongly in favour of the view that the substance producing haemolysis is an amphoteric electrolyte and that both anion and cation are thermolabile, while the undissociated molecules are probably not at all so, under the conditions of the experiment. On this assumption the velocity should be at a minimum when the anions and cations are present in the fewest possible numbers, that is at the isoelectric point. From such experiments therefore

we ought to obtain information about the relative magnitudes of the acidic and basic dissociation constants of the haemolysin. We shall then know whether the haemolysin functions as a weak acid or base in pure watery solution.

The haemolysin I have used is that produced in broth cultures of *Vibrio nasik*. After growing this micro-organism in alkaline broth (such as is employed for the production of diphtheria toxin) for a period of nine or ten days at 37°, it is killed by toluene and the dead bacteria removed by a centrifuge at a speed of about 5000 revolutions per minute. The clear liquid is separated and toluene added. The haemolysin keeps well at room temperature. It is lytic for the red corpuscles of many different animals. I have used a 1 % suspension of sheep red cells in 0.9 % NaCl. The relative amounts of haemolysin present in a series of samples were estimated by Madsen's colorimetric method [Kraus and Levaditi, 1908].

The reaction of nasik lysin was altered by the addition of HCl or NaOH according as an acid or alkaline medium was required. Small amounts of acids simply had the effect of neutralising or lessening its degree of alkalinity—the lysin being alkaline to begin with. In each case a definite amount of acid or alkali was added to 90 cc. of lysin and the whole made up to 100 cc. with distilled water. It was then left overnight in the cold room and next day hydrogen was bubbled through for three hours. This procedure dissipated the traces of toluene which were still dissolved in the lysin, and which Sørensen has shown may adversely affect the electrodes in the electrical method of estimating hydrogen ion concentration. It also had the effect of removing any excess of CO₂ or NH₃. Carbon dioxide is not an electrically active gas, that is to say, it does not give rise to an electromotive force in a platinum electrode dipping in a liquid, but when dissolved in water it forms H₂CO₃ which is slightly dissociated into H⁺ and HCO₃['] ions and therefore increases the acidity of the solution. This would not matter except for the fact that the CO₂ easily diffuses out of the solution, notably into the atmosphere of hydrogen during electrical measurement of the reaction, thereby altering the reaction of the liquid and the saturation of the electrode. Ammonia on the other hand is electrically active and would modify the potential of the hydrogen electrode. In the most alkaline samples of lysin with which I worked there was a very distinct smell of NH₃, due no doubt to decomposition of ammonium salts by the added NaOH and so it was very necessary to get rid of this. The lysin was therefore left in contact with hydrogen for another day in the cold room. The most suitable temperature at which the destruction of the lysin could be followed, was shown by

Famulener and Madsen's experiments to be about 46.0° and this temperature was therefore chosen. Immediately before the heating of the lysin took place, its hydrogen ion concentration $[H']^1$ was measured by the electrical method. The particular type of apparatus used was that described by Michaelis and Rona [1909] the characteristics of which are the *still* atmosphere of hydrogen combined with *minimum immersion* of the electrode. When the electrodes only just dip into the liquid the E.M.F. becomes constant very quickly and this is often of great importance in biological work where the reaction may be altering during the operation. The measurements took place at 46.0° —the same temperature as that at which the destruction of lysin was followed. As normal electrode I used a hydrogen electrode dipping into N/10 HCl. The $[H']$ of this solution at 46° is only slightly less than at ordinary room temperature and it was estimated by graphic interpolation from data given by A. A. Noyes and others [1910] to be dissociated to the extent of 91.4 %. As connecting liquid KCl was used and readings taken with two strengths, namely, 1.75 N and 3.5 N solutions and the true value extrapolated by addition of the difference of the two potentials to the latter according to the method suggested by Bjerrum [1905]. About two minutes was allowed for the cells to attain the temperature of the water bath and then readings taken immediately. Potential equilibrium is practically established by this time as one would expect, especially as the hydrogen electrodes have been in contact with the liquids to be tested for some minutes previous to immersion in the water bath. By continuous observation of the E.M.F. at 46° a very gradual change is perceived due to slight alterations of the reaction of the lysin, as will be mentioned later. An immediate reading as soon as the lysin has reached 46° is necessary therefore and although an error may creep in here it can only be trifling.

The reaction of a given sample of lysin being ascertained, it was next heated to 46.0° . For this purpose a water bath with a toluene regulator was employed. During the heating the temperature never varied more than $\pm 0.025^{\circ}$ and often was practically constant, the thermometer used being an accurate one graduated in tenths of a degree and read with the naked eye. Arrhenius has introduced an empirical formula for the relation between velocity constants and temperature, namely,

$$\frac{k_1}{k_2} = e^{\frac{\mu}{2} \left(\frac{T_1 - T_2}{T_1 T_2} \right)},$$

¹ H' stands for hydrogen ion, the brackets for concentration, and $p_{H'}$ for the negative exponent when the normality is expressed as a power of 10.

k_1 and k_2 being the velocity constants at the absolute temperatures T_1 and T_2 , and μ a constant for a given substance. Famulener and Madsen [1908] have shown that this equation is valid for nasik haemolysin, where $\mu = 128,000$. We can therefore calculate what would be the change in the velocity constant for a rise in temperature from 45.975° to 46.025° . Under these conditions the ratio $\frac{k_1}{k_2} = 1.032$, which represents the maximum error and is so small as to be negligible. The lysin was placed in a large test tube containing the thermometer, which was also used for stirring, and fitted with a large cotton wool plug. It was previously heated to about the required temperature and quickly transferred to the water bath. As soon as the thermometer registered exactly 46.0° a sample was removed by insinuating a narrow bore glass tube at the side of the wool plug, the top of which was fitted to a 10 cc. pipette with a piece of rubber tube. From the pipette 7 cc. were measured off into a tube containing sufficient acid or alkali to neutralise the added alkali or acid, and immediately cooled down in ice-cold water. By this manœuvre the temperature of the lysin in the water bath did not appreciably alter. The attempt to bring the reaction back to its original value by adding the calculated amount of acid or alkali as the case might be, was only partially successful as can be easily imagined, but this is not of great importance since the haemolytic action of the lysin on red cells, within fairly wide limits, is independent of the $[H^+]$. After seven samples had been removed at definite intervals of time the $[H^+]$ of the remainder was determined as before. It was found that in practically every case the reaction had changed during heating to a greater or less extent. It might be thought that it would have been better to alter the reaction by means of regulator mixtures which keep the reaction constant, that is, weak acids or bases in conjunction with their salts, instead of HCl and NaOH. There are however objections to this. In the first place I was desirous of introducing, if possible, only ions of the kind already present. Evidence is accumulating that other anions and cations besides H ions and OH ions, influence the chemical actions of biological substances and so may have altered the chemical relation of the lysin to H and OH ions. In addition, the amount of regulator necessary would be relatively large, and the dilution of the lysin would be such as materially to limit the range over which the destruction could be studied. As can be seen from Fig. 1 the alteration of reaction during heating is in most instances relatively slight and in any case insufficient to vitiate the main results of this investigation. Fortunately the greatest changes took place

in the neighbourhood of the neutral point, where considerable alterations in the reaction produce relatively small changes in the velocity constant.

The following will serve as a type of a single determination of a velocity constant :

	1 N.HCl	H ₂ O	Nasik lysin	[H ⁺]
	2.2 cc.	7.8 cc.	90 cc.	p _H . at 46.0°
				5.47
Heated at 46.0°.				
	Haemolytic Equivalents.		$\left(k = \frac{1}{t_2 - t_1} \log_e \frac{A - x_1}{A - x_2} \right)$	
Minutes	Volumes of lysin producing equal haemolysis		$k \times 0.4343$	
0	0.055			
15	0.065		0.00484	
30	0.07		0.00214	
45	0.072		0.00082	
60	0.075		0.00118	
75	0.09		0.00528	
90	0.11		0.00581	
			Mean	0.00335

To 7 cc. of each sample after heating were added 0.154 cc. of N.NaOH and 0.846 cc. of water. After heating at 46°, p_H. was found to be 5.51.

TABLE I. *Velocity of Destruction of Nasik Lysin at Different Reactions.*

$\left(k = \frac{1}{t_2 - t_1} \log_e \frac{A - x_1}{A - x_2} \right)$ $k \times 43.43$	p _H . at 46.0°	
	before heating	after heating
28.0	8.43	8.49
18.93	8.30	8.23
15.7	8.01	8.17
9.84	8.19	8.10
5.2	7.55	7.62
2.95	7.37	7.17
0.72	7.06	6.95
0.4	7.06	6.62
0.08	6.54	—
0.22	6.46	6.18
0.16	5.82	5.49
0.335	5.48	5.5
2.05	5.17	5.07
3.95	5.04	5.04
6.4	5.10	4.88
10.6	5.00	4.82
16.95	4.77	—

In this way a number of values for k were obtained between two limits of [H⁺] in each case. The results have been tabulated and charted. An inspection of Fig. 1 will reveal the points of interest.

The lines through which the curve has been drawn represent the limits of the change of reaction during heating. The velocity of destruction rises

with increasing acidity and also with increasing alkalinity though not so abruptly in the latter case. The minimum does not correspond with the neutral point but is slightly on the acid side.

Fig. 1. Relation between Velocity of Destruction of Nasik Haemolysin and the Reaction of the Substrate at 46°C.

INTERPRETATION OF RESULTS.

These facts can best be explained on the assumption that the substance causing haemolysis is an amphoteric electrolyte with an acid dissociation constant greater than the basic, that is $k_a > k_b$. If we represent an ampholyte (amphoteric electrolyte) in the usual way

then in strongly acid solution it will be ionised practically entirely thus:

and in strongly alkaline solution

Both the anion and cation are thermolabile and we should expect that the velocity of destruction would be at a minimum at the isoelectric point (I.P.) because here the total number of anions and cations is a minimum. The I.P. is by definition the $[\text{H}']$ of the medium when the anions and cations of the ampholyte are present in equal numbers and it can also be shown that their total concentration is the least possible at a given temperature and in a given solvent—in other words at the I.P. the molecules of an ampholyte suffer least dissociation in solution. It is easy to see that if the H' and OH' have the same tendency to split off from the molecule ($k_a = k_b$) the I.P. will correspond exactly with the neutral point; but if, for example, there is a greater tendency for the H' than the OH' to dissociate, as is the case with so many protein substances, it will be necessary, in accordance with the law of mass action, to add an acid to limit the splitting off of H ions in order that an equal number of anions and cations may be present. Now the neutral point at 46.0° obtained by graphic interpolation from data for the dissociation product of water at different temperatures given by Lundén [1908] is $p_{\text{H}} 6.69$. On reference to Fig. 1 the minimum of the curve appears well on the acid side of the neutral point, and the explanation is that the lysin has a larger acid than basic dissociation constant. These results are consistent with the view that the lysin is an ampholyte comparable with the serum proteins.

There is another possibility, however, namely that the lysin is a dibasic acid. A minimum velocity of destruction would then correspond with a

maximum number of monovalent anions present, and the velocity would increase on the one hand with the number of undissociated molecules of lysin, and on the other hand with the divalent anions, according as the solution was made more acid or less acid respectively.

Trypsin is an ampholyte in which only the monovalent anion has a ferment action, but it seems probable that divalent anions which are inactive are formed by splitting off of another H^+ on further reducing the acidity [Michaelis and Davidsohn, 1911]. Similar considerations would apply in the case of a di-acid base. It is only possible to choose between the two alternatives by testing the direction of movement in an electric field, in solutions of varying $[H^+]$.

II. CATAPHORESIS EXPERIMENTS WITH NASIK LYSIN.

The lysin already employed was not suitable for this purpose on account of its high electrical conductivity. The original broth on which the vibrio was cultivated contained 0.5 % NaCl in addition to salts from the meat and peptone. I first attempted to produce a lysin in a salt-poor medium—peptone water alone or with a little broth added—but I only succeeded in obtaining a weak lysin in this way. Dialysis was next tried. Parchment paper proved useless as the lysin disappeared almost as quickly as the salts from the solution. Much better results followed the use of an animal membrane as dialyser (the so-called Fischblase), which has been successfully used to obtain electrolyte-free ferment solutions. By dialysing for 24 hours against distilled water, I was able to get a fairly strong lysin with an electrical conductivity about quarter that of N/100 NaCl. Reduction of lytic power occurred here also, but to a much less extent than in the case of the parchment membrane.

The apparatus used was that designed by Landsteiner and Pauli, with unpolarisable electrodes of Ag in NaCl, and Cu in $CuCl_2$ which have been recommended by Michaelis. In order to vary the $[H^+]$ a regulator mixture was used as it did not seem probable that the reaction would remain sufficiently constant during the experiment by the simple addition of HCl. It was not necessary to make the solution more alkaline than it already was, as the lysin migrated to the positive pole without any addition.

The most suitable mixture to produce the required range of $[H^+]$ was lactic acid and its sodium salt. This regulator has been used several times in the case of ferments and the lactate ion has not been found to have

any specific effect. This regulator was added to the lysin in the ratio of regulator : lysin = 1 : 9. It is not practicable to dilute the lysin more as its strength would be insufficient to make it easy to detect when it had moved to one or other pole in small quantity. Its content of added salt (sodium lactate) was 0.02 N. This is not very concentrated for a regulator but it seemed desirable to keep the electrolyte content as low as possible. The slaking power of the lysin on the $[H^+]$ of the regulator was considerable. After several mixtures had been made and the values of their $[H^+]$ estimated it was easy to construct a curve from which to predict fairly accurately in advance the $[H^+]$ of any mixture. Such mixtures were put in the middle compartment of the cataphoresis apparatus, and in the side pieces a regulator mixture of as nearly as possible the same $[H^+]$, with the lysin replaced by distilled water. The latter mixture was therefore of slightly lower specific gravity than the lysin mixture, but had the same amount of added electrolyte, if we neglect the slightly dissociated anions of the lactic acid. The electrodes were filled with 0.01 N NaCl in addition to the small amount of concentrated salt solutions—NaCl on the positive side, and $CuCl_2$ on the negative side—which formed layers at the bottom. The whole apparatus when filled was air-tight, and was sunk in a water bath at 10° . A pressure of 200 volts was then applied for four hours. The current passing was of the order of 0.25 milliampere, but varied of course with different conditions of the solutions, the concentration of electrolyte at the electrodes and the size of the electrodes. At the end of the time allowed, 0.4 cc. of liquid was removed from the bottom of each side tube of the apparatus with a Jena glass pipette, which was drawn out to a very fine capillary and was fitted to a syringe by means of a small rubber cork. Very evenly regulated slow suction was produced in this way, so that the bottom layer of liquid just above the taps in the side tubes could be abstracted without mixing with the supernatant layers. To this liquid was added 0.2 cc. of a 3.6 % solution of NaCl, to bring its tonicity up to the level which the red blood cells can tolerate without becoming haemolysed. It was then carefully neutralised with 0.2 N NaOH. The technique of neutralisation presented some difficulties at first owing to the fact that it was not feasible to waste more than a minute amount of liquid in the process, and it was very necessary to bring the solution near neutrality or else haemolysis would be produced by the excess of acid or alkali present. Very small drops were taken and tested with equally small drops of suitable indicators such as methyl-red, rosolic acid, α -naphthol-phthalein, on a white porcelain plate. It was found

absolutely essential to use Jena glass for the pipettes for removal of the liquid and indicators. In this way it was easy to neutralise with a very small total loss of liquid. Lastly 1.0 cc. of a 1 % suspension of sheep red cells in 0.9 % NaCl was added, and the whole incubated for three hours at 37°. The tubes were then shaken and examined next day for haemolysis. A similar sample was always taken from the middle compartment at the end of cataphoresis to show that the lysin had not been destroyed by excessive acidity or other circumstances (see later). In all the mixtures of lysin and regulator a turbidity appeared immediately which gave rise to more or less precipitate as time went on, according to the degree of acidity.

This precipitate is presumably mainly composed of denaturated protein coming from the meat used in the preparation of the original broth, which only completely flocculates when brought to its isoelectric point. At any rate a similar precipitate can be obtained from a meat broth (without peptone) with its optimum at about the same $[H^+]$. The presence of this protein impurity in the lysin solution was a considerable complication. At certain degrees of acidity it had the power of removing much of the lysin from the solution, in the process of precipitation, leaving only a weak lysin to work with. Before discussing its effect on the direction of migration of the lysin I will indicate the experimental results.

Table II shows the direction of movement of the lysin in an electric field at varying $[H^+]$. The temperature was 10° and the $[H^+]$ was determined at the same temperature using in this case a calomel electrode as standard. The π_0 value (0.3383) at 10° was obtained by graphic extrapolation from values given by Sørensen [1912]. It will be seen that in most cases there is not very much difference between the $[H^+]$ of the lysin before and after cataphoresis. The liquid on the + side in every case became more alkaline; that on the - side sometimes increased in acidity but showed less tendency to change from the original reaction of the regulator. It is obvious that these small deviations from the reaction of the lysin in the middle compartment will not militate against the definition of the limit of movement to either pole. For instance a lysin anion travelling towards the + pole on coming in contact with a more alkaline medium will not tend to have its charge reversed as it would if it met a more acid medium, and it will proceed on its way. Similarly on the acid side increased acidity will not interfere with the progress of the lysin cations. The charge and therefore the direction of movement of the ions will thus be determined by the $[H^+]$ of the lysin. It will be seen that movement to both poles has been obtained

TABLE II. *Cataphoresis of Nasik lysin*

Temperature 10° Duration, 4 hours				E.M.F. 200 volts.		k_w at 10°, 10 ^{-14.51}					
No.	Lysin, cc.	0.1 N. Sod. lactate, cc.	0.1 N. Lactic acid, cc.	H ₂ O cc.	p_H				Movement Pole	Remarks	
					Before cataphoresis		After cataphoresis				
					Lysin	Regulator	Lysin (middle compt.)	+ side			- side
1	9	0.5	0.075	0.425	3.92	4.00	3.87	4.23	3.88	+	
2	"	"	0.1	0.4	3.77	3.94	3.85	4.28	3.92	+	
3	"	"	0.15	0.35	3.60	3.60	3.59	3.85	3.63	+	+ liquid turbid.
4	"	"	0.2	0.3	3.29	3.26	3.23	3.55	3.25	0	No movement of visible particles. Strength of lysin after exp. much reduced.
5	"	"	0.4	0.1	3.01	2.87	3.01	3.02	2.9	0	
6	"	"	0.5	0	2.94	2.99	2.92	3.10	2.97	-	- liquid turbid.
7	"	"	0.5	0	2.94	2.70	—	2.9	2.78	-	
8	"	"	0.7	0	2.75	2.73	2.82	3.02	2.74	?	All lysin destroyed at end of exp., due to extreme acidity.

with a zone of no movement between them, according to the degree of acidity. This points to the fact that the lysin is an ampholyte and lends additional support to the interpretation of the experiments on velocity of destruction of the lysin by heat.

DISCUSSION.

The early experiments to determine the electric charge of antigens (toxins, etc.) were all vitiated by neglect to exclude sharp changes of reaction at the electrodes. Field and Teague [1907] used platinum electrodes and in addition complicated their work by the presence of agar, through which they designed the toxin should travel and lend itself to easy detection. They determined that both the toxin and antitoxin of diphtheria and tetanus had positive charges which were not reversed by altering the reaction of the solvent—a most unlikely result in the light of later research. Teague and Buxton [1907] believed that they had shown that haemagglutinins moved to the cathode and considered that “all the active principles of serum concerned in the anti-reactions have a tendency to migrate towards the cathode.” Their experiments were carried out in a similar way to those of Field and Teague except that they used unpolarisable electrodes. Bechhold [1907] used membranes with the idea of excluding the products formed at the poles from the substance being examined.

Landsteiner and Pauli [1908] in the case of abrin, ricin, and the haemagglutinin found in normal fowl serum, obtained a movement to the negative pole in each. A determination of the $[H^+]$ of the solution was here lacking, but it was presumably not far from the neutral point. These experiments are an advance on the previous ones as the fallacies of the method were eliminated.

Landsteiner [1913] states that he has some unpublished results showing that staphylolysin and arachnolysin are amphoteric. The only details given are that in weak acid reaction the lysins migrated to the negative pole, and in weak alkaline to the positive pole.

Let us now attempt to co-ordinate the two series of results, namely, the curve of velocity of destruction and the movement in an electric field. The temperature in the two cases was different because the minimum velocity of destruction of the lysin had to be reduced for the cataphoresis experiments, so that the lysin should not be appreciably weakened by the end of the time allowed. Suppose for the present we estimate the I.P. at 10° (Table II) at about p_H 3.3. At 46° the minimum velocity of destruction

can be read from the curve at roughly $p_H \cdot 6.1$. Is it reasonable to suppose such a difference in the I.P. would occur at the two temperatures? In the first place it must not be forgotten that values of $[H']$ for different temperatures are not directly comparable. For instance at 46° the $[H']$ of neutral reaction is $p_H \cdot 6.69$, but at 10° the $[H']$ of neutral reaction is $p_H \cdot 7.26$.

It is obvious that these two values are thermodynamically equivalent. It is in fact the ratios H'/OH' , which correspond at two different temperatures, this ratio being of course equal to unity at neutrality. A simple calculation shows that the ratios of H' to OH' will be the same at the two temperatures when the p_H is 6.1 at 46° and 6.67 at 10° . In other words there is a difference of I.P. corresponding to the difference between $p_H \cdot 6.67$ and $p_H \cdot 3.3$ to be accounted for. We have now to see what is the effect of temperature on the other amino-acids. The only data I have been able to find in the literature are those of Lundén [1906]. He determined the acid and basic dissociation constants of three simple amino-acids at 25° and 40° . I have calculated the shift of I.P. in each case, by means of the equation $R = \frac{I^2}{k_w}$

[Michaelis and Rona, 1910].

Ampholyte	Temp- erature	k_a	k_b	$\frac{k_a}{k_b} = R$	Iso-electric Point	
					$R = \frac{I^2}{k_w}$ p_H	at $25^\circ = 10^{-14}$ at $40^\circ = 10^{-13.19}$
Acetoxime	25°	6.0×10^{-13}	6.5×10^{-13}	0.92	7.02	
	40°	9.9×10^{-13}	19.0×10^{-13}	0.52	6.89	
β -i-Asparagine	25°	1.35×10^{-9}	1.53×10^{-12}	882.4	5.53	
	40°	3.22×10^{-9}	4.23×10^{-12}	761.2	5.56	
<i>o</i> -Amino-benzoic acid	25°	1.06×10^{-5}	1.38×10^{-12}	7.68×10^6	3.56	
	40°	1.35×10^{-5}	3.16×10^{-12}	4.37×10^6	3.68	

The basic constant in each case increases more than the acidic with rise of temperature, which leads to a displacement of the I.P. towards the alkaline side. In the case of acetoxime the basic constant is greater than the acidic and the I.P. is consequently on the alkaline side of neutrality, but in the last two the I.P. is on the acid side. Allowing again for the fact that the values of I.P. need correction as they are determined at different temperatures, which correction as we have already seen still further increases the difference, we find that the nett result of a rise of temperature on the I.P. in the case of ampholytes like β -i-asparagine and *o*-amino-benzoic acid, is to shift it towards the neutral point. This is exactly what has taken place with the lysin. It is true that the difference in the last case is much greater than

the more extended range of temperature would account for and it is quite possible that there are other factors concerned. It must be remembered, for instance, that the salt content of the lysin solution in the heat destruction experiments was very different from that in the cataphoresis experiments. However the lysin is in all probability much more complicated than such simple ampholytes as the ones cited, so that it is unwise to expect too close a parallel. From this point of view therefore the experimental facts are in harmony with the theory that the lysin is an ampholyte.

We have still to consider the effect of the presence of other substances on the behaviour of the lysin. I have already drawn attention to the fact that it is not pure. In biochemistry we are constantly faced with the difficulty that the body we are investigating is mixed with other substances which may influence its action. There is some experimental evidence bearing on this point. Firstly there are some anomalous instances of heat destruction of haemolysins. Madsen, Famulener, and Walbum [1904] found that the haemolysin of staphylococcus was greatly reduced in strength at 70°, but almost completely returned on raising the temperature to 100° for five minutes. Landsteiner and Rauchenbichler [1909] found the same thing and attributed it to a union of staphylolysin at a medium temperature with some other substance in the solution, which compound was dissociated again at 100°. Working with megatheriolysin I was able to show [Atkin, 1910] that the velocity of destruction increased up to a temperature of about 55°, then it decreased up to a temperature of 75°, after which it increased up to 100°. This phenomenon is probably due to the same cause as that operating in the case of staphylolysin. I also repeated Madsen and Famulener's experiments without being able to confirm their results—the staphylolysin showed only a gradually increasing destruction with rise of temperature. This seems further to substantiate the view that the return of activity at a high temperature is not a characteristic of the lysin itself, but due to the influence of some foreign substance. Naturally in different samples the quantitative if not the qualitative relations of the several constituents will vary. No such property exists in the case of nasik haemolysin however, possibly because of its relatively great thermolability, for it is completely destroyed at a temperature considerably less than that at which the supposed union takes place in the case of staphylolysin and megatheriolysin. Similar considerations also apply to tetanolysin. If the hypothesis that megatheriolysin undergoes a union with some other body at certain temperatures, which modifies its destruction by heat, is correct, the process of destruction

Fig. 2. Destruction of Megatheriolysin.

→ Logarithms of lysin concentration

The points of origin of the curves are arbitrarily chosen for convenience of presentation.

should not be a unimolecular one. In other words, if we plot the logarithms of lysin concentration against time we ought not to get a straight line. If, on the other hand, at higher or lower temperatures such a combination either does not take place or is disrupted, it is reasonable to expect the process to be then a logarithmic one. I have applied this test to my experimental results in the case of megatheriolysin [Atkin, 1910] and find it actually to be so.

As the curves show (Fig. 2) the process of destruction is only unimolecular, firstly from a temperature of about 74° , upwards, which is near the point at which the rate of destruction again begins to increase with temperature; secondly, below a temperature of about 43° . It is not quite certain that the destruction at 40.0° is logarithmic, but at any rate it approximates closely to a straight line, if it is in reality a curve. It has been drawn in the figure as a dotted line. This last point is interesting as it shows that the influence of the foreign substance is being felt at a temperature considerably lower than that at which it is able to decrease the velocity of destruction with rising temperature. It will be noticed that the hypothetical union with another substance is not a reversible process, otherwise the decomposition which presumably takes place at high temperatures would result in a re-union on cooling down, for we must remember that the actual test on red blood cells is carried out at 37° . In fact the observation that we are able to detect the phenomenon at all renders it highly probable that it is irreversible, for if this were not so we should expect the union at medium temperatures to be split up again on cooling to 37° . We seem to have a criterion therefore with regard to the abnormal destruction of lysins by heat, namely, that if at any temperature it can be shown that the destruction is *not* unimolecular, the velocity of destruction will not rise regularly with temperature, or what is another phase of the same phenomenon, the strength of the lysin will decrease at a certain temperature, and reappear again at a higher one.

In my present experiments with nasik lysin the temperature was kept constant at 46° , the reaction alone being changed. It is hardly to be expected that a union with another body would take place having the same effect as in the case of megatheriolysin, seeing that this effect requires a considerably higher temperature, but nevertheless I have subjected the figures relating to the velocity of destruction to the same test and find that in every case the process is unimolecular. This fact is in favour of the chemical freedom of nasik lysin, under the conditions of these experiments.

Work bearing on the influence of impurities on the movements of enzymes in an electric field has been carried out by Pekelharing and Ringer [1911]. One of them devised a method of purifying crude pepsin by dialysing and thus reducing the acidity, so that the enzyme partially separates out. They do not claim that their product is absolutely pure, but at any rate it is more nearly pure than any commercial preparation. With it they obtained migration to the anode only, within the limits of acidity they tried. Addition of caseinogen, crystallised serum albumin, or albumose (Witte's peptone), caused migration more or less to the cathode. The pepsin was in fact carried by the albumin, etc. They were of opinion that the value 5.5×10^{-3} found by Michaelis and Davidsohn [1910] for the I.P. of pepsin was not reliable.

In the present case the lysin is mixed with, at any rate, one other protein substance which flocks out at a definite $[H^+]$. It is difficult to say whether the lysin also flocks out at its I.P. as its absolute concentration must be extremely small and at 10° the I.P. apparently nearly coincides with that of the protein impurity. A glance at Table II, No. 4, shows that at this point there is no movement of the visible particles and at the same time the solution has become depleted of most of its lysin. We may here have a condition of things where the lysin and visible precipitate have opposite electric charges and consequently a union has taken place. The lysin would then presumably move with the precipitate. If this were so this boundary of the I.P. or rather isoelectric zone of the lysin would be rendered obscure. When however the sign of the charge of the lysin changed, the two bodies would again have similar charges and the lysin would be free to move independently. Similar considerations apply to the other boundary because the visible particles may become negatively charged before the lysin. A rough approximation to an isoelectric zone is therefore all that can be attained. If we take this zone as p_H 3.0 to 3.6 and assume that the geometric mean represents the I.P. then this will be the arithmetic mean of the p_H values, namely p_H 3.3. Therefore approximately,

Isoelectric point at 10° p_H 3.3.

,, ,, 46° p_H 6.1.

This represents a large decrease in acid character with rise of temperature. It is interesting to attempt a determination of the acid and basic dissociation constants. Michaelis and Menten [1913] have devised a graphic method of obtaining these constants. Using the velocity constants I have constructed

dissociation-residue curves but find that they do not correspond with any theoretical curve, the steepness being much greater. The action of the H' and OH' cannot be one of simple control of the dissociation of the lysin, but they must also take part in the destruction by heat. It is impossible therefore from the data to determine the dissociation constants, but the I.P., where H' and OH' are in equal numbers, gives us a measure of the ratio of acid to basic constant.

From this investigation we can conclude that the experiments relating to heat destruction are in favour of the view that nasik haemolysin is an ampholyte. This is confirmed by the cataphoresis experiments. I must admit, however, that the presence of other proteins may influence the results, at any rate of the cataphoresis experiments. Similar objections may be raised to the whole series of the many determinations of the direction of movement in an electric field in the case of ferments, which have been investigated in the last few years, and it is quite possible that all these results will have to be revised.

SUMMARY.

1. The velocity of destruction of nasik haemolysin at 46.0° is at a minimum slightly on the acid side of the neutral point. This is in favour of the view that the lysin is an ampholyte with its acid dissociation constant greater than its basic, and the point of minimum destruction is the isoelectric point. The anion and cation are therefore thermolabile, and the undissociated molecules not so.

2. Cataphoresis experiments at 10° confirm this view.

3. Approximate values for the isoelectric points are

$$p_{H'} \text{ at } 10^\circ = 3.3; \quad p_{H'} \text{ at } 46^\circ = 6.1.$$

This decrease of acidity with rising temperature is also found in the case of simple ampholytes, but to a smaller extent.

4. Incidentally a criterion is given for the anomalous heat destruction of certain haemolysins, for instance, megatheriolysin, namely, that the process is not unimolecular at temperatures where the velocity of destruction decreases with rising temperature, but it can be shown to be so at temperatures considerably removed where the velocity of destruction follows the more ordinary course of increase with rise of temperature.

REFERENCES.

- Atkin (1910). *Zeitsch. Immunitätsforsch.*, **7**, 656.
Bechhold (1907). *München. med. Woch.*, **54**, 1921.
Bjerrum (1905). *Zeitsch. physikal. Chem.*, **53**, 428.
Famulener and Madsen (1908). *Biochem. Zeitsch.* **11**, 186.
Field and Teague (1907). *J. Exp. Med.*, **9**, 86.
Kraus and Levaditi (1908). *Handbuch d. Techn. u. Method. d. Immunitätsforschung*, **1**, 57.
Landsteiner (1913). Article on *Kolloide und Lipide in der Immunitätslehre*. Kolle and Wassermann's *Handbuch der pathogenen Mikroorganismen*.
Landsteiner and Pauli (1908). *Verhandl. 25th Kongr. f. Innere Med. Wien.*, 571.
Landsteiner and Rauchenbichler (1909). *Zeitsch. Immunitätsforsch.*, **1**, 439.
Lundén (1906). *Zeitsch. physikal. Chem.*, **54**, 532.
— (1908). *Affinitätsmessungen an Schwachen Säuren und Basen*, 45. Bd. xiv aus der Sammlung chemischer und chemisch-technischer Vorträge. Ferdinand Enke. Stuttgart.
Madsen, Famulener and Walbum (1904). *British Med. J.*, **2**, 572.
Michaelis and Davidsohn (1911). *Biochem. Zeitsch.*, **36**, 280.
— (1910). *Biochem. Zeitsch.*, **28**, 1.
Michaelis and Menten (1913). *Biochem. Zeitsch.*, **49**, 333.
Michaelis and Rona (1909). *Biochem. Zeitsch.*, **18**, 317.
— (1910). *Biochem. Zeitsch.*, **27**, 38.
Noyes, A.A., and others (1910). *Zeitsch. physikal. Chem.*, **70**, 335.
Pekelharing and Ringer (1911). *Zeitsch. physiol. Chem.*, **75**, 282.
Sørensen (1912). *Ergebnisse d. Physiol.*, **12**, 393.
Teague and Buxton (1907). *J. Exp. Med.*, **9**, 254.