Onderstepoort Journal of Veterinary Science and Animal Industry, Volume 1, Number 1, 1933.

Chemical Blood Studies.*

II. A contribution to the determination of Urea in Animal Blood Filtrates ("Laked" and "Unlaked").

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In the course of studies (Graf, 1933) on the composition of blood of domestic animals subjected to various South African stock diseases it was found that the method of Folin and Svedberg (1930) for the determination of urea gave most inconsistent results. For the precipitation of the protein matter in the laked blood 10 c.c. of a 0·725 N sulphuric acid and 10 c.c. of a 11 per cent. sodium tungstate solution were used, as against 10 c.c. of a 0·66 N sulphuric acid and 10 c.c. 10 per cent. sodium tungstate as prescribed by Folin and Wu (1919). This change became necessary since it was found that with some blood samples the proteins were not completely precipitated when using the prescribed amounts of reagent. The unlaked blood, on the other hand, was treated strictly according to standard procedure. After various preliminary experiments, and after checking up the reagents employed most carefully, only two possible sources of error remained to be considered, viz. :—

I. —That the degree of acidity of the blood filtrates was such that the 2 c.c. of saturated borax solution employed was not sufficient to liberate on distillation all the ammonia derived from the urea. It should be pointed out here that, on account of the presence of amino-acids and other amino-derivatives contained in the blood filtrate, no strong alkali can be used for liberating the ammonia, as amino substances would thus be subjected to partial hydrolytic decomposition with the liberation of additional ammonia.

II.—That, as a result of the work by other investigators on the activity of the urease enzyme, and more specially as the result of the valuable study by Barendrecht (1919) concerning the effect of hydrogen ion concentration on this activity, it became evident that too great a fluctuation in the pH of the medium would result in erroneous urea determinations. In Folin and Svedberg's method it is suggested to regulate this apparently fluctuating degree of acidity of medium by buffering with two drops of acetate buffer.† Whether by this means an optimum pH for urease activity will be achieved obviously depends on the degree of acidity (or alkalinity) of the initial blood filtrate.

* The titles of the series will be found under "References."

† Dissolve 15 gm. of crystallized sodium acetate in a 100 c.c. volumetric flask by the help of 50 to 75 c.c. of water. Add 1 c.c. of glacial acetic acid (about 99 per cent.), dilute to volume, and mix.

Measuring the pH of such animal blood filtrates by the potentiometric method, using the quinhydrone electrode, it was found that these filtrates varied appreciably in pH, although always distinctly acid in reaction. In the table below are given the limiting values of pH of blood filtrates obtained from blood drawn from normal and infected animals of different species.

Animal.	Number of cases.	Laked or unlaked.	pH Fluctuation.
Sheep.....	20	Laked.....	3·01—4·30
	24	Unlaked.....	3·75—4·59
Bovine.....	8	Laked.....	2·98—4·13
	8	Unlaked.....	3·79—5·06
Blesbok (*Damaliscus albifrons*)...	6	Laked.....	2·71—3·69
	6	Unlaked.....	3·81—4·23

It would appear, therefore, that the acidity of blood filtrates varies greatly, ranging at least from pH 2·71–5·06.

I.—The Liberation of Ammonia by means of Borax.

In order to investigate the first possible source of error, a series of solutions of different pH and containing 2·5 c.c. of a standard solution of ammonium carbonate (5·0 mgm. N/100 c.c.) was prepared by adding varying quantities of dilute sulphuric acid (N/20) and diluting up to a final volume of 8 c.c. In all cases the two drops of acetate buffer solution, as recommended by Folin, were added. The pH of these solutions, and the amounts of ammonia recovered after distilling with borax and Nesslerization have been tabulated in Table I.

Table I.

	c.c. N/20 H_2SO_4 added.	pH of Soln. with buffer.	Borax Soln. added.	pH of Soln. after adding borax.	NH_3 found as N.
1.....	0·0 c.c.....	5·45	2·0 c.c..	ca. 9·2	0·138
2.....	0·2 „	5·25	2·0 „ ..	„ 9·2	0·138
3.....	0·6 „	5·03	2·0 „ ..	„ 9·1	0·147
4.....	1·0 „	4·81	2·0 „ ..	„ 9·0	0·138 *Blank*=
5.....	2·0 „	4·20	2·0 „ ..	„ 8·9	0·138 0·013
6.....	3·0 „	2·76	2·0 „ ..	„ 8·8	0·138 *mgm N*
7.....	4·0 „	2·37	2·0 „ ..	—	0·147
8.....	3·0 „ N/10	1·90	2·0 „ ..	—	0·092
9.....	3·0 „ N/10	1·90	4·0 „ ..	—	0·100

It is clear, therefore, that down to a pH of 2·4 the 2 c.c. of borax solution added is sufficient to liberate all the ammonia quantitatively. As it is also highly improbable that the acidity of a blood filtrate will rise to a pH below this limiting value, it may be concluded that the 2 c.c. of saturated borax solution added will in every instance liberate quantitatively all the ammonia present as such at the time of distillation.

II.—The Regulation of pH and Urease Activity.

Having shown that the 2 c.c. borax solution proved adequate for the final liberation of the ammonia, attention was next diverted to the urease conversion of the urea into ammonium carbonate. The urease used was derived from the Soja or Jack bean by grinding up the beans, and extracting in the cold with ca. 30 per cent. aqueous alcoholic solution using 100 c.c. of this diluted alcohol per 30 gm. soja-bean meal. The extract was absorbed by filter paper (NH_3-free) which was then dried and cut up into rectangular pieces approximately 1 × 2.5 cm.

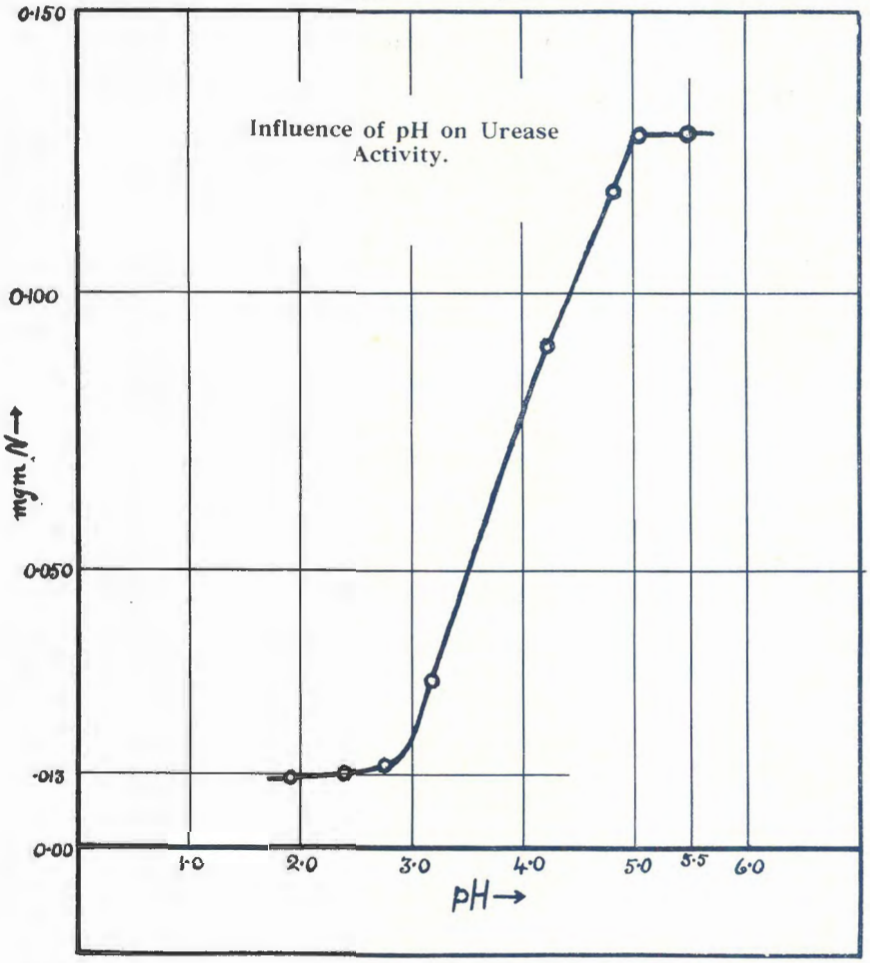
For the purpose of studying the effect of pH on the activity of the urease, a series of solutions of varying pH and containing 2·5 c.c. of a standard urea solution (5·0 mgm. N/100 c.c.) was prepared by adding the necessary amount of N/20 sulphuric acid, the two drops of acetate buffer, and diluting to a final volume of 8 c.c. To each of these solutions two pieces of the urease paper were added and the solutions allowed to stand from two to four hours with occasional shaking. The results, obtained in duplicate, have been tabulated in Table II.

Table II.

Experiment.	c.c. N/20 H_2SO_4.	pH of sol. without buffer.	pH of sol. with buffer.	Reaction time with urease.	c.c. Borax added.	NH_3 found, as mgm. N.	
						Duplicate values.	Average.
1.....	0·0 c.c.	ca. 8·0	5·45	2 hrs. 5 min.	2·0 c.c.	—	0·128
2.....	0·5 „	„ 2·54	5·05	2 „ 25 „	2·0 „	0·125 0·128	0·127
3.....	1·0 „	„ 2·30	4·81	3 „ 5 „	2·0 „	0·121 0·115	0·118
4.....	2·0 „	„ 2·03	4·20	3 „ 15 „	2·0 „	0·080 0·100	0·090
5.....	3·0 „	—	2·76	3 „ 35 „	2·0 „	—	0·014
6.....	4·0 „	„ 1·81	2·37	3 „ 55 „	4·0 „	0·015 0·011	0·013
7.....	3·0 „ *N/10*	„ 1·66	1·90	4 „ 15 „	4·0 „	0·012 0·011	0·012

From these results it would appear that in experiments 5–7 the activity of the urease is zero, since the ammonia found corresponds almost exactly with the blank values derived from Table I. However, the marked effect of pH on the activity of the urease is best illustrated in the accompanying graph, obtained by plotting the weight (in mgm.) of recovered N against the pH of the medium. We note that with an acetate buffer the urease activity reaches an optimum at an acidity within the pH limits 5·0 and 5·5. Below pH 5·0 there is a very sudden fall in activity resulting ultimately in stagnation. It is clear, therefore, that the urease can effect a quantitative conversion of urea into ammonium carbonate only if the acidity of the medium is carefully regulated and kept within the optimum pH range of 5·0 to 5·5.

From Table II it is also evident that the addition of two drops of acetate buffer does not necessarily result in the attainment of this optimum condition. On the other hand, the pH of the buffer solution itself must lie somewhere near 5·5, since such solutions do not appreciably alter in pH on dilution. Hence the pH of all solutions, irrespective of their initial acidity, must eventually be brought to fall within this optimum range if sufficient buffer solution is



added. That this desired effect can easily be achieved in the actual course of analysis is shown by the results tabulated in Table III.

TABLE III.

c.c. n/20 H_2SO_4 added.	Initial pH (o.o. c.c. buffer).	pH 2 drops buffer.	pH 4 drops buffer.	pH 8 drops buffer.	pH 10 drops buffer.	pH 20 drops buffer.
0·0 c.c.....	ca. 8·0	5·45	—	—	5·43	5·37
2·0 „	„ 2·03	4·20	4·67	4·95	5·05	5·15
4·0 „	„ 1·81	2·37	—	—	4·81	5·05

The solutions used in the above experiment were obtained by adding the specified amounts of n/20 sulphuric acid to 2·5 c.c. of the standard urea solution, then adding the required amount of buffer solution and finally diluting to 8 c.c. Hence, starting with an initial pH as low as 1·8 and adding 20 drops of the acetate buffer, the pH of the resulting medium is brought to within the required optimum range. Again, as the highest acidity found in blood filtrates was of the order of pH 2·7, it may be concluded that the addition of 20 drops buffer solution should prove adequate in all cases. Folin and Svedberg's (1930) urea determination method must therefore be modified if serious errors are to be avoided. It is suggested that instead of two drops of acetate buffer, 1·5–2·0 c.c. of this buffer solution be used per 5 c.c. blood filtrate.

Applying the method in its thus modified form, and using 1·5 c.c. buffer solution per 5 c.c. aliquot blood filtrate, good results are obtained as Table IV readily shows.

TABLE IV.

Animal.	Laked or unlaked.	pH of blood filtrate.	Urea found as mgm. n/100 c.c. blood.
Sheep No. 26689.....	Laked.....	3·63	20·0
	Unlaked.....	4·27	19·4
Bovine No. 3532.....	Laked.....	3·93	12·2
	Unlaked.....	4·31	11·4
Blesbok No. 32055.....	Laked.....	2·71	12·6
	Unlaked.....	3·81	12·2
Blesbok No. 32054.....	Laked.....	3·45	19·0
	Unlaked.....	3·83	17·4

Since this modification was introduced hundreds of urea determinations have been made in this laboratory in the course of studies (Graf, 1933) on animal blood. In no case was any further difficulty encountered.

REFERENCES.

BARENDRECHT, H. P. (1919). Urease en de stralingstheorie van enzymewerking I en II. Rpt. of General Meeting. Wis- en Natuurkundige Afdeling der Koninklijke Akademie van Wetenschappen (Amsterdam) 22 Feb. 1919 en 29 Maart 1919, Part 27.

FOLIN, O., AND WU, H. (1919). A system of blood analysis. *Jnl. Biol. Chem.*, Vol. 38, p. 81.

FOLIN, O., AND SVEDBERG, A. (1930). An improved distillation method for the determination of urea in blood. *Jnl. Biol. Chem.*, Vol. 88, p. 77.

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GRAF, H. I. Comparative studies on blood, "laked" and "unlaked" blood filtrates of animals in health and disease, with particular reference to methods and technique employed.

JORDEN, T. J., AND GRAF, H. II. This article.

GRAF, H. III. Comparative studies on "laked" and "unlaked" blood filtrates of sheep in health and during Heartwater and Bluetongue.

GRAF, H. IV. Comparative studies on "laked" and "unlaked" blood filtrates of horses in health and during Horsesickness.

GRAF, H. V. Comparative studies on "laked" and "unlaked" blood filtrates of cattle in health and during Anaplasmosis and Piroplasmosis.