CCXXXVII. THE OPTIMUM BUFFER p_H FOR HYDROLYSIS OF UREA BY UREASE, AND THE PREPARATION OF STABLE UREASE POWDER.

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WHEN estimating urea by their improved technique Kay and Sheehan [1934] found that hydrolysis of urea in aqueous solution was not always complete, especially with small amounts, whereas under identical conditions hydrolysis of urea in blood appeared always to be complete; stronger extracts of soya beans also gave more nearly complete hydrolysis. Both blood and soya bean extracts are slightly alkaline. It appears probable therefore that the 0.6 % KH_2PO_4 solution used as buffer is too acid in reaction for complete hydrolysis of urea in aqueous solution. When this buffer is partly neutralised by adding a quarter of its equivalent quantity of NaOH , estimations show almost complete hydrolysis of even small amounts (*e.g.* 0.5 mg.) of urea in aqueous solution. These facts suggest that a systematic investigation of the effect of the buffer used would be of value, especially as a technique capable of high accuracy is now available. For this purpose a stable urease preparation is needed. Attempts to prepare crystalline urease by Sumner's method [1926] failed.

In the present paper we give an account of the preparation of a dry, stable urease powder, and of an investigation of the effects of different phosphate buffers on the efficiency of the hydrolysis of urea in aqueous solution by this urease, with a view to defining the optimum buffer p_H .

The preparation of a stable urease powder.

Finely ground and sifted jack bean meal was digested at room temperature for one hour with five times its weight of distilled water. Then by straining through fine cotton gauze and thoroughly centrifuging the liquid, a homogeneous extract was obtained. This was concentrated to a syrupy consistency under reduced pressure at 25–29° with gentle agitation by a fine, slow stream of CO_2 bubbles, which also prevented frothing and bumping. 140 ml. of concentrate (from 500 ml. of original extract) were poured into 500 ml. of acetone, with brisk stirring, the resulting precipitate being filtered off on a suction-filter and washed with acetone.

One portion of the sticky, rubbery mass obtained was dried *in vacuo* over concentrated sulphuric acid at room temperature, and yielded a hard, horny mass which was powdered. Another portion was ground with pure dry acetone and became a coarse, granular powder, which, after filtering, washing with dry acetone and drying in air at room temperature, was easily ground to a fine powder. The second method of treatment is preferable as it is quicker and less

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laborious. Subsequent tests of activity showed that the two powders were of almost identical strengths; they were therefore mixed and became the stock source of urease. In all, 47 g. of dry powder were obtained from 1000 ml. of original aqueous extract.

The final powder is slightly yellowish in colour and dissolves readily in water or phosphate buffer solutions to give an opalescent solution. It has a faintly acid reaction (0.02 % solution has p_H 6.5–6.6) and exercises only a slight buffering effect. It is so nearly free from ammonia that “blank” estimations in the aeration-titration method of estimating urea represent alkaline spray only. It has shown no evidence of deterioration after 10 months in the laboratory without special storage precautions.

Tests of activity of the urease powder.

1. *Comparison with original jack beans.* Different amounts of urease powder in aqueous solution were incubated for 10 minutes at 50° with 5 ml. of 3 % urea solution in phosphate buffer of p_H 6.4. The reaction was stopped by addition of mercuric chloride and the ammonia formed titrated. The same procedure was carried out with different volumes of a jack bean extract, prepared as described above.

Equivalent hydrolysis of urea was given by 50 mg. of urease powder and by 5 ml. of jack bean extract (equivalent to 1.0 g. of meal), which hydrolysed 138 and 139 mg. of urea respectively. The activity of the powder is thus about twenty times that of the meal. Since the yield of powder is rather less than 25 % of the weight of meal it thus appears as if the isolation process not only concentrates the urease but actually increases its hydrolytic power.

2. *Hydrolytic power of the stock urease powder.* Using phosphate buffers of different p_H values, 0.5 mg. of stock urease powder was allowed to act in this case for 30 minutes at 50° on 150 mg. of urea. The reaction was stopped by adding K_2CO_3 and the ammonia formed estimated by aeration and titration. The following results were obtained:

At p_H	mg. urea hydrolysed	At p_H	mg. urea hydrolysed
5.8	3.4	6.8	4.7
6.0	4.3	7.0	3.1
6.4	4.7	7.2	2.7

Under the most favourable conditions of p_H , the powder hydrolyses more than nine times its own weight of urea, whilst even under the most adverse conditions, at p_H 7.2, it hydrolyses at least five times its own weight of urea. This is stronger than the powder prepared by Van Slyke and Cullen's method [1914] but much weaker than Sumner's recrystallised urease, which had about 1000 times the activity of the jack beans from which it was prepared [Sumner and Hand, 1928].

It was considered therefore that in subsequent estimations 2 mg. of urease powder would be a safe excess for the quantities of urea used (0.5–6 mg.). Results proved this to be so.

The effect of buffer p_H on the efficiency of urea hydrolysis.

Known amounts of pure urea, varying from 0.5 to 6.0 mg. were estimated in duplicate, using 2 mg. of stock urease powder and phosphate buffers varying in p_H from 4.5 to 8.4. The technique and apparatus were exactly as described by Kay and Sheehan [1934] except that a larger volume of air was used for aeration.

Pure urea was prepared by recrystallising dry, chemically pure urea from pure dry acetone and finally drying *in vacuo* over concentrated H_2SO_4 for 12–16 hours before use. Solutions of appropriate strength in distilled water were made as required, so that the desired amount of urea could be exactly measured in accurately calibrated 1 or 2 ml. blood pipettes.

The phosphate buffers were made by mixing $M/5$ solutions of KH_2PO_4 and NaOH according to the standard tables of Clark [1928]. 5 ml. were used in each estimation.

A 1 % stock solution of urease powder in p_{H} 7.0 phosphate buffer was made. This keeps under ordinary laboratory conditions for at least a week, but actually was renewed at shorter intervals. It was diluted as required by appropriate buffers so that the resulting solutions contained 2 mg. of powder in 5 ml., the volume used in the estimations. The p_{H} of the buffer solutions was not appreciably affected by the urease.

The total volume of urea-urease-buffer mixture in each estimation was always 12 ml., 1 ml. of distilled water being added when only 1 ml. of urea solution was used.

The results are set out in Table I, the averages of the paired estimations being given. The agreement between members of each pair compared favourably with that obtained by Kay and Sheehan [1934], the average difference between members corresponding to 0.022 ml. $N/75$ NaOH . In Table I account is not taken of the small portion of ammonia which aeration fails to transport, but as this is almost constant the figures are comparable amongst themselves. Owing to the larger volume of air used in aeration as compared with that employed by Kay and Sheehan, in the present work values of 98–99 % may be taken to represent complete hydrolysis. Tests of the alkaline liquid after aeration constantly showed small amounts of untransported ammonia.

Table I. *Efficiency of hydrolysis of different amounts of urea at varying buffer p_{H} values.*

Urea used mg.	0.5	1.0	1.5	2.0	4.0	6.0
p_{H} of buffer	Percentage of urea estimated					
4.5	7.0	9.8	12.2	14.8	54.9	78.1
4.7	15.6	17.6	28.1	35.5	75.7	93.8
5.8	58.0	66.6	78.5	85.3	91.3	94.6
6.2	94.5	94.3	97.0	97.9	94.4	93.9
6.3	—	—	—	—	97.7	98.1
6.4	93.9	95.8	98.4	98.7	98.5	98.0
6.6	96.8	97.9	99.0	97.8	98.3	98.9
6.8	96.5	97.7	99.0	98.7	98.0	98.2
7.0	96.1	97.1	98.4	98.7	98.6	99.2
7.2	95.8	98.2	99.0	98.6	98.1	98.9
7.6	—	—	—	—	98.3	98.3
7.8	96.6	97.9	98.7	98.8	96.9	94.3
8.0	—	—	—	—	97.0	87.4
8.4	94.2	94.4	97.1	96.3	71.5	71.0

The poor efficiency with buffers of low p_{H} is apparently due to the medium being too acid. On the other hand, the fall in efficiency with large amounts of urea and buffers of high p_{H} is to be attributed to the medium becoming too alkaline for urease to work satisfactorily. Van Slyke and Zacharias [1914] said that although the neutral point is the optimum p_{H} , with decreasing amounts of urea the p_{H} should increase to obtain optimum conditions.

Evidently for general purposes, for estimating urea in aqueous solutions or in blood, an initial reaction of p_H 6.6–7.0 is most satisfactory. This range is given by KH_2PO_4 solution to which one-third to one-half of its equivalent of NaOH is added, the final solution being adjusted to represent a concentration of 6 g. per litre of original KH_2PO_4 . Thus to 6 g. KH_2PO_4 dissolved in a little distilled water add 0.6–0.88 g. NaOH and make up to 1 litre. This buffer is very similar to that recommended by Peters and Van Slyke [1932]. The optimum buffer p_H values and the relative efficiency of hydrolysis for different amounts of urea are set out in Table II, again ignoring the small amount of untransported ammonia.

Table II.

Actual urea mg.	Optimum p_H range of buffer	Average % of urea hydrolysed	Actual urea mg.	Optimum p_H range of buffer	Average % of urea hydrolysed
0.5	6.6–7.8	96.3	2.0	6.2–7.8	98.5
1.0	6.6–7.8	97.8	4.0	6.3–7.6	98.2
1.5	6.2–7.8	98.5	6.0	6.3–7.6	98.5

SUMMARY.

1. A method of preparing a dry stable urease powder from jack beans is described.
2. The optimum initial p_H values for buffers in the urease hydrolysis of urea have been determined for amounts of urea varying from 0.5 to 6.0 mg.
3. For general purposes, including blood-urea estimations, a safe initial p_H range is 6.6–7.0 for quantities up to 6 mg. of urea.
4. This range is obtained by adding to KH_2PO_4 solution one-third to one-half of its equivalent of NaOH and adjusting the solution to contain the equivalent of 0.6 % of KH_2PO_4 .

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